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(54) Title: NONTHROMBOGENIC GLYCOSAMINOGLYCAN COPOLYMERS (57) Abstract New biocompatible glycosaminoglycan copolymers which are antithrombotic and antithrombogenic, are provided for demanding biomedical applications requiring long-term or permanent maintenance of anticoagulant properties. The novel copolymers of the present invention are comprised of small fragments or segments of glycosaminoglycans such as heparin, copolymerized with synthetic monomeric components. The invention takes advantage of the fact that small segments of heparin, or other glycosaminoglycans, possess and retain antithrombotic activity that retards or prevents the thrombosis. The anticoagulant fragments are conveniently produced by enzymatic or chemical means and copolymerized with synthetic monomers.		

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NONTHROMBOGENIC GLYCOSAMINOGLYCAN COPOLYMERSTechnical Field

The present invention relates to synthesis of glycosaminoglycan copolymers which are biocompatible and nonthrombogenic in vitro and thus can be used in biomedical devices.

Background Art

The importance of the clinical application of devices or materials which contact blood is well recognized in the practice of modern medicine. Generally speaking, all materials used in biomedical devices need to be biocompatible. The use of biomedical devices has frequently been limited due to adverse reactions between the foreign surface and one or more components of blood, a major restriction being the propensity of foreign surfaces to support thrombosis.

Use of anticoagulants has helped minimize the problem of thrombosis in extracorporeal devices, but it often in turn causes complications such as a hemorrhagic tendency when used systemically (Porter, J., and Tick, H., J Am Med Assoc (1977) 237:879-881). These problems have prompted the research for heparin substitutes and analogs, but these have shown consistently lower anticoagulant activities than heparin (Casu, B., Adv Carbohydrate Chem Biochem (1985) 43:51-134).

Considerable effort has also been devoted to developing blood-compatible materials from which devices could be fabricated. Approaches to retard surface thrombosis on materials include tailoring a polymer

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surface to minimize interactions or selectively adsorb a passivating albumin layer. Additional efforts have been made to incorporate pharmacologically active agents into the polymer via bulk dispersion or by surface immobilization of anticoagulants. Other approaches include creating biological coatings or pseudoneointimal lining for intravascular prosthesis. But a synthetic surface has not previously been produced which would permanently resist thrombus formation.

In recent years, numerous reports have described the efforts devoted to the synthesis of polymers with improved biocompatibility. Blood compatibility of polyurethanes have recently been reviewed (Ito, Y., and Imanishi, Y., in Critical Reviews in Biocompatibility, CRC Press, 5:45-104, 1989). Various physical properties of synthetic polymers have been examined to determine how they relate to thrombotic potential of biomaterials, but to date no one or combination of these properties is predictive of biocompatibility (Addams, G.A., Transplantation/Implantation Today (1986) 3:45-56).

Attempts have been made to avoid the hazards associated with systemic use of heparin while exploiting its anticoagulant properties. Surface heparinization of biomaterials can render materials nonthrombogenic. The slow release of heparin ensures anticoagulant protection but the supply is eventually exhausted (Imanishi, Y., in Current Topics in Polymer Science (R.M. Ottenbrite, et al., Eds), vol. I, Hansen Publishers, Munich, 1987, pp. 58-78).

Attempts to attach heparin to surfaces have also been met with limited success. Ionic attachment of heparin onto polycationic surfaces of polymers have been prepared (Tanzi, M.C., et al., in Polymers in Medicine II (E. Chiellini et al., Eds.) Plenum Press, New York, 1986,

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pp. 67-85 and 91-99). Heparin can also be attached by covalent or cross-linking to various surfaces.

(Jozefowicz, M., and Jozefowicz, J., in Polymers in Medicine II (E. Chiellini et al., eds.) Plenum Press, New York, 1986, pp. 41-65). Improved biocompatibility has been reported for heparin-polyvinylalcohol hydrogel used for coating of various surfaces (Ip, W.F., et al., J Biomed Mater Res (1985) 19:161-176). These materials are only suitable for use in devices for short term applications. There are problems due to inadequate potency, limited incorporation, degradation by plasma heparinase, sequestering by heparin neutralizing species, and lack of platelet inhibitory effect.

In a completely different approach, a chemical or radiochemical treatment of heparin induces the generation of a macro-radical which in conjunction with a monomer results in a covalently incorporated heparin copolymer. However, these may or may not have an anticoagulant activity and are rather poor compared to intact heparin (Boffa, M.C., et al., Thromb Haemostas (1979) 41:346; Salzman, E., et al., in Chemistry and Biology of Heparin (R. Lundblad et al., Eds.) Elsevier, New York, 1980, p. 435).

Other synthetic polymers which have been heparinized in attempts to produce better nonthrombogenic surfaces, have also met with limited success. A new antithrombogenic polymer was synthesized by the photograft-copolymerization of methoxypolyethyleneglycol methacrylate and dimethylaminoethyl methacrylate to polyacrylonitrile, followed by heparinization of the graft copolymer (Miyama, H., et al., J Biomed Mater Res (1986) 20:895-902). Surfaces of commercial polyurethanes have been modified by poly(ethylene oxide) grafting and heparin immobilization for long-term biomedical applications (Han, D.K., et al., J Biomed

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Mater Res Appl Biomater (1989) 23(A1):87-104: Park, K.D., et al., Trans. 15th Annual Soc. Biomater. (1989) 12:26). Similarly, random copolymers of 2-hydroxyethyl methacrylate-styrene (Lea, S., et al., Trans 15th Annual Soc Biomater (1989) 12:25) and amphophilic block copolymers of poly(dimethylsiloxane) and poly(ethylene oxide) containing heparin (Grainger, D.W., et al., J Biomed Mater Res (1988) 22:231-250; Piao, A.Z., et al., Trans 15th Annual Soc Biomater (1989) 12:28) have been prepared via a series of coupling reactions using functionalized prepolymers, diisocyanates, and derivatized heparins. Some of these have been achieved with considerable difficulty associated with a sequence of derivatizations and also at the expense of antithrombin III cofactor activity of heparin.

Other compounds displaying anticoagulant activity have been described in the literature. The inhibition or prevention of platelet adhesion on polymers has been attempted by means of combining prostaglandin-heparin conjugates with polymers (Kim, S.W., Artif Organs (1987) 11:228-236). Unfortunately, prostacyclin is extremely unstable in plasma, not to mention that it is very costly and difficult to produce. A number of polymeric materials with some antithrombogenic activity were prepared by binding sulfonate, carboxylic, amino acid sulfamide or amide groups onto cross-linked polystyrene, polysaccharides or polystyrene-polyethylene graft copolymers (Jozefowicz, M., et al., in Polymers in Medicine II, pp. 41-65, 1986). The anticoagulant activity of these materials was found to be dependent on the content and nature of the substituting groups, but they were generally much less effective than heparin (Mauzac, M., and Jozefowicz, J., Biomaterials (1984) 5:301).

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Recently, a new polymer comprising essentially a backbone having a polyurethane structure carrying pendant groups containing phosphatidylcholine or homologues thereof has been described (Chapman, D., and Valencia, G.P., US Patent 4,689,386, 1987) to mimic the outer surface of the lipid matrix of naturally occurring biological membranes (Hayward, J.A., and Chapman, D., Biomaterials (1984) 5:135-142). Grafting or deposition of phosphorylcholine onto cellulose acetate membranes via radiation techniques (Jayasree, G., and Sharma, C.P., Trans 15th Annual Soc Biomater (1989) 12:185) and a copolymer of phosphorylcholine and methylmethacrylate have also been reported (Nakabayashi, N., et al., *ibid*, 12:9, 1989). However, it is well known that platelet phospholipids, viz. alkylacylphosphatidylcholine can be converted enzymatically into platelet-activating factor which is an extremely potent platelet stimulating factor.

A nonthrombogenic surface prepared by covalent bonding or grafting of heparin fragments via a modified reducing terminal residue (reductive amination) has been described (Larm, O., et al., Biomater Med Dev Artif Organs (1983) 11:161). However, incorporation of oligosaccharides derived from heparin into polymeric backbone has not been previously attempted.

There is considerable evidence in the literature showing that a necessary structural prerequisite for significant heparin anticoagulant activity is a specific pentasaccharide sequence of heparin that constitutes the minimal binding site for antithrombin (Casu, B., Adv Carbohydrate Chem Biochem (1985) 43:51-134). This specific heparin segment is sufficient for the antithrombin-mediated inhibition of Factor Xa but inadequate for the effective inhibition of thrombin and for the development of full anticoagulant activity. In fact, the antithrombin-mediated inhibition

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of thrombin requires a segment of at least four (trisulfated) disaccharide units in addition to the pentasaccharide sequence of the active-site for thrombin (Lane, D.A., et al., Biochem J (1984) 218:725-732). However, the sizes of these heparin fragments are far from those of conventional heparin which may contain a considerable proportion of higher molecular weight fractions, the latter being responsible for inducing platelet adhesion/aggregation as well as binding to other plasma components. Therefore, incorporation of smaller heparin segments with anti-Xa activity into a polymeric backbone would provide a permanently antithrombotic and chronically nonthrombogenic (thromboresistant) or antithrombogenic biomaterial.

The advantage of using lower amounts of heparin segments would be to prevent thrombosis while reducing or eliminating the hemorrhagic risks associated with high anticoagulant activity of conventional heparin. A biomaterial incorporating such heparin fragments into a polymeric backbone would be useful for the prevention of surface thrombosis in a more efficient manner since the surface would be less susceptible to competing interactions with various heparin-neutralizing or degrading species including lipoproteins, fibrinogen, etc., which are more abundant than the antithrombin in plasma. This surface would also be less prone to platelet adhesion and promotion of their aggregation or interaction with such cell surface components as fibronectin and laminin. In addition, the material would provide a negatively-charged nonthrombogenic surface, somewhat similar to that of a vascular endothelium, and a pseudo-affinity for antithrombin by mimicking the array of essential sulfate groups in the binding site for antithrombin, yet inappropriate for thrombogenic interaction with fibrinogen or platelets.

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Disclosure of the Invention

There is a continuing need and much research for materials usable in biomedical devices which come into contact with blood, wherein these devices are thromboresistant and antithrombotic. Two basic approaches have been employed--one approach involves simultaneous or contemporaneous administration of an anticoagulant, such as heparin, to prevent initiation of thrombosis at the surface of the medical device. Another approach involves derivatizing heparin or other anticoagulants directly to the portions of the device which come into contact with the blood. The latter approach often employs fairly complex derivatization procedures. Furthermore, both of the foregoing approaches are limited by the lack of long-term stability of heparin under physiological conditions.

The present invention solves these problems by including anticoagulant and antithrombotic glycosaminoglycan fragments as monomeric units in copolymers with non-glycosaminoglycan monomeric units that confer the physical properties required for construction of the device. Thus, the devices may be constructed, in those portions which contact the blood, with the copolymer which itself has anticoagulant, nonthrombogenic, and antithrombotic properties. In addition, these copolymers may be used to coat surfaces of devices intended for blood or other biological contact. Finally, water soluble copolymers are useful as pharmacological agents for the treatment of coagulation disorders or of certain immunological, cardiovascular and viral diseases.

The copolymers of the invention can also be synthesized in the presence of crosslinkers to provide forms of the copolymers with more rigid physical properties, if desired. The copolymers can also be

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further derivatized to additional moieties by employing monomeric units which contain functional groups that permit further derivatization. This derivatization can include derivatization to, for example, affinity ligands, label, or additional glycosaminoglycan units to enhance the anticoagulation effect.

Thus, in one aspect, the invention is directed to a biologically compatible copolymer suitable for the construction and/or coating of biomedical devices which contact the blood. The copolymer is constructed by copolymerization of at least one monomeric unit which is a glycosaminoglycan fragment capable of conferring the desirable antithrombotic, nonthrombogenic, and anticoagulant properties along with at least one additional non-glycosaminoglycan monomeric unit which confers the suitable physical properties required for the construction or coating of the device. Of course mixtures of glycosaminoglycan fragments and/or mixtures of copolymerizing monomeric units can be used to form the copolymers. The physical characteristics desired in the resulting copolymer will determine the choice of the nonglycosaminoglycan monomeric unit. The physical characteristics are also affected by the type and amount of crosslinking employed. Water-soluble forms of the copolymers are also included in the invention, as are derivatized forms of these copolymers.

In another aspect, the invention is directed to a method to prepare the copolymer of the invention which method comprises mixing the individual monomeric units or their components under conditions wherein copolymerization takes place.

In still another aspect, the invention is directed to medical devices constructed and/or coated with the copolymer of the invention.

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Brief Description of the Drawings

Figure 1 shows the degradation of heparin and typical products from (A) nitrous acid cleavage and (B) heparinase digestion.

Figure 2 shows copolymerization schemes for acrylamide with typical (A) heparin fragment generated by heparinase digestion (Example 1), (B) allyl glycoside derivation of product from heparinase digestion, and (C) aminoethylmethacrylate derivative of nitrous acid cleavage product (Example 2).

Figure 3 shows the proton NMR spectrum in D_2O of a "putative copolymer" of acrylamide with heparin decasaccharide obtained from heparinase digestion (Preparation A). The copolymer (designated CH5-139, prepared by Example 1) contains 7.9 weight % heparin fragment after purification by desalting on a Sephadex G-25 column, followed by dialysis against 50 mM EDTA and finally against Type I water. Inset shows proton NMR spectra of corresponding heparin decasaccharide.

Figure 4 shows a typical proton NMR spectrum in D_2O of a copolymer of acrylamide with aminoethylmethacrylate derivative of heparin octasaccharide produced by limited deaminative cleavage with nitrous acid (Preparation B). The copolymer (CH8-32B, prepared by Example 2) contains 28 wt % heparin fragment, following purification on a Sepharose 4B column eluted with 0.2 M NaCl and dialysis against pure water.

Figure 5 shows the proton NMR spectrum in deuterated DMSO of a polyurethane-type polymer prepared by copolymerization of tolylene diisocyanate and ethylene glycol with low molecular weight heparin produced from nitrous acid cleavage. The copolymer (CF3-22C, prepared by Example 6) contains 0.7 wt % heparin after extensive washing with chloroform, ethanol and finally with water until free from unreacted heparin and other monomers.

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Figure 6 shows the proton NMR spectrum in deuterated DMSO of the copolymer of acrylonitrile with an allyl derivative of heparin hexadecasaccharide (from heparinase digestion) containing 8% (by weight) of heparin, prepared in Example 11.

Figure 7 shows a typical FTIR spectrum of a polyurethane-type copolymer, as prepared in Example 18, of tolylene-2,4-diisocyanate and Tris-(hydroxymethyl) aminomethane-derivatized, low molecular weight heparin (4,000-6,000 Da). The copolymer contains 5.24 µg heparin/mg of solid.

Figure 8 shows copolymerization schemes for the synthesis of copolyurethanes with low molecular weight heparin fragments.

Modes of Carrying Out the Invention

The invention is directed to copolymers which are useful in construction and/or coating of medical devices which contact blood. These copolymers are advantageous because their biocompatibility properties are such that they can be used to contact blood without the thrombotic effects usually evidenced when blood contacts nonbiocompatible surfaces.

As used herein, "nonthrombogenic" means that the material does not itself cause the formation of thrombi or the coagulation of blood. "Antithrombotic" and "anticoagulant" mean that the materials not only do not themselves cause clotting but actively prevent the formation of blood clots encouraged by other stimuli. The copolymers of the invention are both antithrombotic and nonthrombogenic.

These properties are conferred by incorporation into the copolymer of antithrombotic and nonthrombogenic fragments of glycosaminoglycans. The fragment is generally prepared by depolymerization of a suitable

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glycosaminoglycan, but the possibility of synthetic preparation of these fragments is by no means excluded. At the present time, preparation through depolymerization is more economic.

Glycosaminoglycans (GAGs) are biological polymers, generally sulfated, which are repeating disaccharide units of uronic acid-glycosamine disaccharides. The category in which the GAG is classified is dependent on the nature of these subunits. Heparin is perhaps the best known GAG, and consists mostly of repeating iduronic acid-glucosamine subunits which are sulfated at some of the available hydroxyl and amino positions. Dermatan sulfate is comprised of disaccharide units which are either iduronic or glucuronic acid coupled to a galactosamine residue, also sulfated at some of the amino and hydroxyl positions. Other GAGs useful in the invention include hyaluronic acid, heparan sulfate, and chondroitin sulfate, especially types A and C.

The GAGs, regardless of their nature, are susceptible to depolymerization using appropriate enzymes or by the use of chemical degradation. For example, heparin is conveniently degraded by heparinase digestion; chondroitin sulfate by chondroitinase. Chemical degradation is most commonly conducted using nitrous acid or periodate. The nature of the degradation products will depend on the mode of degradation. Furthermore, the nature of the degradation scheme will also determine the availability of functional groups for further polymerization in the product low molecular weight GAG.

In general, if the reducing terminal aldehyde is retained, this can be derivatized after the degradation to obtain an allyl derivative which can participate in addition polymerization with unsaturated non-GAG monomers. Additional carbonyl moieties may also be generated by certain degradation schemes, and these

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can be further derivatized or used directly in the polymerization reaction. Available carbonyl groups can also be reduced to the alcohols which can participate in polymerization reactions with isothiocyanates. Some degradation schemes directly generate carbon-carbon unsaturation in the sugar residues. The available functional groups in the degradation products can also, if desired, be provided with linkers which attach suitable functional groups for polymerization reactions.

An illustrative scheme for the degradation of GAGs is shown for heparin in Figure 1. As seen in Figure 1A, degradation in nitrous acid results in deamination and ring contraction of the reducing terminal glucosamine residue to obtain a 2,5-anhydromannose. The resulting free aldehyde at the reducing terminus is a convenient reactive group for derivatization to materials capable of forming copolymers. Periodate degradation would also generate aldehyde functionalities. This free aldehyde may also be reduced to obtain the corresponding anhydromannitol.

As shown in Figure 1B, heparinase degradation results in formation of the 4,5-D-uronate at the nonreducing terminus which can be used directly in addition polymerization.

The foregoing is not to imply that either the aldehyde or uronate functionality must be employed directly or indirectly in formation copolymers. However, use of these functionalities limits the copolymerization to the site of these groups whereas utilizing, for example, a free hydroxyl or carboxy group would involve the internal saccharide residues of the fragment in the polymer coupling.

Figure 2 illustrates some of the modalities for the copolymerization of the GAG fragments with additional monomeric units. As shown in Figure 2A, for example, the

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4,5-uronate resulting from heparinase digestion can be reacted with acrylamide to obtain an addition polymer having a heparin fragment integrated into the resulting copolymer. As shown, multiple acrylamide residues may (and typically will) form the "monomeric unit" with which the glycosaminoglycan fragment is copolymerized. Thus, as used herein, "monomeric unit" refers respectively to the glycosaminoglycan and non-glycosaminoglycan subunits of the copolymer which will be, of course, nonuniform, in a typical copolymerization. Thus, it is theoretically possible that the heparinase fragments could dimerize or trimerize by virtue of the unsaturation at the nonreducing terminus; it is extremely likely that oligomer formation of the acrylamide components will take place. Thus, "component" is used to refer to an individual monomer; "monomeric unit" is used to refer to an individual GAG or non-GAG region of the copolymer.

Figure 1B shows a different approach for providing unsaturation in the GAG fragment for participation in addition polymerization. The reducing terminus can be conveniently derivatized to an unsaturated moiety, such as here shown in the formation of an allyl glycoside. Formation of allyl derivatives is standard in the art. These then can copolymerize with a non-GAG monomeric unit as illustrated here, with acrylamide. Again, formation of oligomeric units as the defined "monomeric unit" from the acrylamide occurs. While the illustration shows involvement of the allyl group in the polymer, it is of course possible that polymerization will take place at the nonreducing terminus as well, in a manner analogous to that shown in Figure 2A.

Figure 2C illustrates still another approach involving derivatization of a nitrus acid degradation product of heparin through amination of the aldehyde to

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provid the desired unsaturation, in this case to the aminoethylmethacrylate. The unsaturation in the methacrylate is then a convenient site for copolymerization with the additional monomeric units supplied by acrylamide.

As illustrated in Figure 2, the functionality appropriate for inclusion of the GAG fragment in an addition copolymer can be supplied by derivatization of a convenient functional group on the fragment. Thus, in general, a C-C double bond can be derivatized to the reducing terminus of the fragment by virtue of the reactivity of the aldehyde or hemiacetal. An allyl group can be derivatized directly as described in the figure, or a C-C double bond containing residue, such as acrylic acid or a derivative thereof, can be coupled with the free aldehyde or the open chain form at the reducing end. In addition, C-C double bonds residing in the GAG per se as a result of degradation at the nonreducing terminus can be used directly in the copolymerization.

In addition to the examples illustrated in Figure 2, addition polymers can be formed using diisocyanates as shown in Figure 8. In this instance the appropriate reactive group is hydroxy or amino. Addition is to the N-C double bond of the isocyanate. As the GAG fragment will have, in many cases, a multiplicity of hydroxyl groups, this method is most advantageous when a primary alcohol can be supplied to react preferentially in comparison to the mostly secondary hydroxyls in the fragment. The products of nitrous acid depolymerization provide an aldehyde which is readily reduced to a primary alcohol using, for example, a borohydride. For heparin depolymerization, this results in a 2,5-anhydromannose which can then be conveniently reduced to the corresponding anhydr mannitol. For dermatan sulfate depolymerization, the product is a 2,5-anhydr talose

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which can be reduced to the corresponding anhydrotalitol. The primary alcohol provided by the reduction is available for reaction with isocyanate so that the fragment is incorporated by addition polymerization.

The foregoing copolymerization may also be modified by including in the non-GAG "monomeric unit" a diol which polymerizes initially with the diisocyanate. Thus, for example, ethylene glycol, propylene glycol, 1,3-propanediol and the like can be included in the copolymerization mixture, resulting in copolymers of the diol with the diisocyanate as well as inclusion of the GAG fragment.

As will be apparent from the foregoing discussion, the non-GAG "monomeric unit" used in the copolymer can be composed of a variety of monomers, dimers, trimers, and oligomers wherein these monomeric units may be homogeneous with regard to the monomers which comprise them or which may be heterogeneous. Candidates for components which comprise the non-GAG monomeric units in the copolymers of the invention include acrylic and methacrylic acid and their carboxyl derivatives such as the amides and esters thereof. The amides may be the primary amide as in the illustration of Figure 2 or may be, for example, an alkyl amide of 1-6C. Similarly, the esters may be the alkyl esters of 1-6C or the alkyl groups of the ester may be substituted, preferably with hydrophilic substituents such as hydroxyl. Thus, suitable as components of the non-GAG monomeric unit are the hydroxyalkyl esters, the aminoalkyl esters, and the glyceryl esters of acrylic or methacrylic acid. Also useful in the invention are other hydrophilic alkylenes such as esters of vinyl alcohol, allyl alcohol, acrylonitrile, and the like. The ester groups of the vinyl alcohol esters can be hydrolyzed, if desired, after polymerization. In some instances, less

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hydrophilic non-GAG components, such as styrene, styrene sulfonic acid, or propylene can be used.

In general, if the non-GAG subunit is formed from an unsaturated monomer, the monomer component will have the general formula $\text{CH}_2=\text{CXY}$ wherein X is H or CH_3 and Y is selected from the group consisting of $-\text{COOR}$, $-\text{CONH}_2$, $-\text{CONHR}$, $-\text{CONR}_2$, $-\text{CONH}(\text{CH}_2)_n\text{OH}$, $-\text{CONH}(\text{CH}_2)_n\text{OR}$, $-\text{COO}(\text{CH}_2)_n\text{OH}$, $-\text{COO}(\text{CH}_2)_n\text{OR}$, $-\text{OCOR}$, $-\text{OR}$, $-\text{Cl}$, $-\text{CN}$, $-\text{C}_6\text{H}_5$, substituted $-\text{C}_6\text{H}_5$, $-\text{OCO}(\text{CH}_2)_n\text{OH}$, $-\text{OCO}(\text{CH}_2)_n\text{OR}$, $-\text{OCO}(\text{CH}_2)_n\text{NH}_2$, $-\text{OCO}(\text{CH}_2)_n\text{NHR}$, and $-\text{OCO}(\text{CH}_2)_n\text{NR}_2$, wherein n is an integer of 1-4 and R is a straight or branched chain alkyl group of 1-4C. Further reaction may be conducted with respect to Y after polymerization occurs, e.g., $-\text{OCOR}$ may be hydrolyzed to $-\text{OH}$. A preferred value of n is 2; preferred embodiments of R are methyl and ethyl.

The non-GAG "monomeric unit" may comprise copolymers of a multiplicity of these components. Thus, for example, a copolymer of hydroxyethyl methacrylate and a vinyl alcohol ester could be used. As used herein, "multiplicity" means that two or more different GAG or non-GAG components are used either with respect to a monomeric unit, the copolymer as a whole, or both.

As stated above, the non-GAG fragment can also be a urethane type of monomeric unit. Urethane-type units are obtained by condensation of a diol with a diisocyanate. Useful diisocyanates include tolylene-diisocyanate, and diisocyanate derivatives of the various alkylene diamines.

The copolymerization of the GAG and non-GAG subunits will be conducted under conditions suitable for the particular components chosen. The non-GAG fragment may be preoligomerized or, more typically and preferably, the components of the fragment may be supplied as the monomers in the copolymerization mixture. General

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conditions for such copolymerizations are known to those in the art.

As stated above, either a single type of GAG or non-GAG component may be used, or a multiplicity of either or both. Thus the GAG component G1 may be copolymerized with the non-GAG component NG1 to obtain copolymers, e.g., of the form:

$$\text{-G1-(NG1)}_3\text{-G1-(NG1)}_5\text{-G1}$$

or

$$\text{-NG1-G1-NG1-G1-NG1-}$$

or

$$\text{-G1-(NG1)}_{10}\text{-(G1)}_2\text{-(NG1)}_3\text{-}$$

Alternatively, two different (a multiplicity) of GAG components, e.g., G1 and G2 may be used to obtain, for example:

$$\text{-G1-(NG1)}_3\text{-G2-(NG1)}_{10}\text{-G2-}$$

or, two different (a multiplicity) of non-GAG components can be used to obtain, for example:

$$\text{-G1-(NG1)}_3\text{-G1-(NG1-NG2-NG1-NG2)-G1}$$

or

$$\text{-G1-(NG2)}_4\text{-G1-(NG1-NG2-NG2-NG1)-G1}$$

or

$$\text{G1-(NG2-NG1)-G1-(NG2-NG2-NG1-NG1)-G1}$$

and the like.

Typical molecular weights of the resulting copolymer are sufficiently high that the appropriate physical characteristics will be conferred to allow the construction of the desired medical devices. Such devices include catheters, delivery lines for

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extracorporeal treatment, endoscopic instruments, and the like. Also, water-soluble copolymers in particular can be used as pharmacological agents for the treatment of coagulation disorders or certain immunological, cardiovascular and viral diseases.

Crosslinkers

The copolymer may also be formed in the presence of a crosslinker to obtain a product with physical properties which may be more desirable for the particular use intended. Typical crosslinkers for addition-type polymers include monomer components with two centers of unsaturation, such as bis forms of acrylamide or of the hydroxyalkyl acrylate esters. The crosslinking moiety is included in the polymerization mixture a weight percent of about 5-15% of the non-GAG monomer component, preferably about 10% of the non-GAG monomer component.

Derivatization of the Copolymers

The copolymers can further be derivatized to other useful moieties, including, for example, affinity ligands, label or, in particular, additional low molecular weight GAG. For such derivatization, it is convenient to supply monomers for formation of the copolymer which contain additional functional groups, unreactive under the conditions of polymerization, but capable of reacting thereafter with functional groups in the moiety to be derivatized to the copolymer. For example, use of aminoethyl methacrylate in the non-GAG portion provides free amino groups, which can derivatize with the aldehyde groups of low molecular weight heparin. Any convenient coupling strategy can be employed depending on the nature of the substance to be

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derivatized and the functionality of the resulting copolymer.

Finally, in some instances, the backbone copolymer contains only non-GAG components and includes low molecular weight GAG as an intact derivatized ligand. In this case, also, functional groups inert to the polymerization are included in the monomeric components and then used to bind selectively at only one functional group, for example, the reducing terminus, of the GAG. This form of the invention is illustrated below in Examples 13-15, 17 and 19-21.

Description of the Preferred Embodiments

The following examples are given to illustrate, but not to limit the invention.

Preparation A: Degradation of Heparin with Heparinase from Flavobacterium Heparinum and Derivatization of Heparin Fragments into Allyl Glycosides

A. Heparin is digested with Flavobacterium heparinase according to the method of A. Grant et al. (Anal Biochem (1984) 137:25-32). One gram of commercial porcine mucosal heparin (Sigma) is dissolved in 5.0 mL of 25 mM sodium acetate, 0.25 mM calcium acetate, pH 7.0. 1250 units of commercial heparinase from Flavobacterium heparinum (also, from Sigma) is dissolved in 5.0 mL of 50 mM sodium phosphate, pH 7.4. These two solutions are combined, mixed well and allowed to incubate overnight in a 30°C water-bath. The enzymatic degradation product is monitored periodically by measuring the absorbance of 20 µl of digestion mixture in 2.0 mL of 30 mM HCl at 232 nm.

After incubation for approximately 20-24 h, the digestion mixture is divided into 5 x 2 mL aliquots (200 mg heparin in each). Each aliquot is loaded

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separately on a series of two 100 cm x 2.5 cm Sephadex G-50 superfine columns (Linker, A., and Hovingh, P., Biochemistry (1972b) 11:563-572). Fifteen minute fractions are eluted in 0.2 M NaCl, 10% ethanol at a flow rate of 0.4 mL/min. All fractions are assayed for heparin by measuring their absorbance at 232 nm, and resolved into distinct peaks corresponding to different sizes of heparin fragments (disaccharides to hexadecasaccharides) in the elution profile. The heparin fragments in the column fractions are then dialyzed separately against Type I water and lyophilized.

B. Derivatization of heparin fragments into allyl glycoside: 450 mg of the fragment to be derivatized is dried completely under vacuum with a cold trap in line for 24 hours. This is dissolved in water-free allyl alcohol and then added to cationic (acidic) Amberlite resin (1R-120H) which has been prepared by washing it 5 times with water, methanol, allyl alcohol and finally water-free allyl alcohol. This mixture is allowed to react at room temperature while stirring vigorously. After 96 hours, the reaction mixture is filtered and the resin is washed 5 times with 50:50 ethanol:water and 98% ethanol. The filtrate and washings are pooled, concentrated to a small volume, and loaded onto a reverse phase silica gel column (20 cm x 1.5 cm). The column is washed sequentially with 50 mL water, 50 mL methanol:H₂O fraction containing the allyl glycoside of the heparin fragment is then lyophilized.

Preparation B: Depolymerization of Heparin by
Deaminative Cleavage with Nitrous Acid

Depolymerization of heparin is carried out according to the procedure of B. Casu et al. (Biochem J. (1981) 197:599-609). One gram of commercial porcine mucosal heparin is dissolved in 400 mL of Type I water to

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make a 0.25% solution. This solution is brought to 0.1 M H_2SO_4 by adding to it 2.14 mL of concentrated H_2SO_4 . The mixture is cooled to 10°C in an ice-bath and then made 0.2 M in NaNO_2 by dissolving 5.55 g NaNO_2 in it. This is allowed to react for 2 min at 10°C. The solution is then neutralized by adding 10 M NaOH until the pH stabilized at 7.0-7.2. The mixture is concentrated and partially desalted using a 500 molecular-weight-cut-off Amicon membrane, and lyophilized. The dried heparin fragments are then redissolved in a small volume of Type I water and resolved on Sephadex G-50 superfine columns, as described in preparation A. Fractions are assayed for heparin content using the uronic acid assay of T. Bitter and H.M. Muir (Anal Biochem (1962) 4:330-334). The heparin fragments of different size (disaccharides to hexadecasaccharides) present in the column fractions are dialyzed separately against Type I water and lyophilized.

Example 1

Copolymerization of Acrylamide With Heparin Fragments Containing 4,5-Unsaturated Uronides Generated by Heparinase Digestion

A. A typical polymerization mixture contains 10 mg of a heparin fragment (hexa- to hexadecasaccharide) with the 4,5-unsaturated bond (prepared as described in paragraph A of preparation A), 11.6 mg of acrylamide, and 0.725 μL TEMED (N,N,N',N'-tetramethylethylenediamine), in 180 μL water. This mixture is degassed for 30 min before addition of 0.18 μg ammonium persulfate in 5 μL water. Polymerization is allowed to proceed 18 h at 4°C. The copolymer is separated from monomers, including any unreacted heparin fragment, on a Sephadex G-25 desalting column, and/or by dialyzing extensively against 50 mM EDTA. Heparin containing fractions are detected by the carbazole method (Bitter and Muir, 1962) for uronic acid

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analysis. Fractions are pooled, dialyzed finally against Type I water and lyophilized. NMR spectra confirms the presence of both polyacrylamide and heparin in the product, as shown in Figure 3.

B. Using the same general procedure as set forth in paragraph A of this Example, a number of copolymers were prepared with heparin fragments of different size, listed as follows:

<u>Copolymer Designation</u>	<u>Fragment/Size of Heparin Used</u>	<u>% Uronic Acid in Copolymer</u>	<u>% Heparin Fragment in Copolymer</u>
CH6-131D	Octasaccharide	3.6	13
CH6-131E	Decasaccharide	7.3	26
CH6-131F	Dodecasaccharide	10.4	39

Example 2

Copolymerization of Acrylamide With Aminoethylmethacrylate Derivative of Heparin Fragments Generated by Nitrous Acid Cleavage

A. 1.25 mL aqueous solution (20 mg/mL) of low molecular weight heparin (LMWH), obtained by deaminative cleavage with nitrous acid (Sigma H5640, average molecular weight 4,000-6,000 Daltons), is warmed to 50°C in a water bath. To this is added 1.25 mL of an aqueous solution containing 6.25 mg of 2-aminoethyl methacrylate (AEM, Kodak 18513). Derivatization of heparin proceeds at 50°C for 1 h with stirring.

B. Copolymerization with acrylamide is accomplished by the addition of a 2.5 mL solution of acrylamide in water (BioRad 161-0100, 56 mg), degassing, then adding 50 µL N,N,N',N'-tetramethylethylenediamine (TEMED, Bi Rad 161-0880) and 10 mL of a stock solution of ammonium persulfate (Bi Rad 161-0700, 0.5 mg/µL).

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Polymerization continues with stirring at 50°C for 10 min. A two times molar excess of NaBH₄ over AEM is then added, and reduction of the aldehyde-imine bond is carried out for 30 min at room temperature. Separation of copolymer from unreacted monomers is achieved by chromatography on a column of Sepharose 4B, 1.5 cm x 80 cm, eluted with 0.2 M NaCl at a flow rate of 0.27 mL/min. The copolymer peak is detected by assaying 15 min fractions for uronic acid by the carbazole method (Bitter and Muir, 1962). Copolymer elutes close to the total volume, V_t, of the column. NMR spectra confirms that the copolymer is composed of both heparin and polyacrylamide, a typical example being shown in Figure 4.

C. Following the same procedures of paragraphs A and B above, but using different heparin fragments obtained from preparation B described earlier, several copolymers were prepared as listed below:

<u>Copolymer Designation</u>	<u>Fragment/Size of Heparin Used</u>	<u>% Uronic Acid in Copolymer</u>	<u>% Heparin Fragment in Copolymer</u>
CH8-23D	LMWH (Sigma)	6.8	22
CH8-32B	Octasaccharide	9.5	28
CH8-36A	Decasaccharide	10.0	35

Example 3

Copolymerization of Styrene With Aminoethyl-methacrylate Derivative of Heparin Fragments From Deaminative Cleavage With Nitrous Acid

Low molecular weight heparin (Sigma, H5640, average molecular weight 4,000-6,000 Daltons) is derivatized with aminoethylmethacrylate (AEM). 36 mg of heparin is dissolved in 1.8 mL of water to make a 2%

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solution which is warmed to 50°C. 1.8 mL of a 5 mg/mL AEM solution is added to the warm heparin solution. This is allowed to react for 1 hour at 50°C.

42 mg of AEM-derivatized heparin is dissolved in 240 mL of formamide by warming to 70°C. The resulting heparin solution is made 0.1% in sodium dodecyl sulfate (SDS) by adding 0.27 mg SDS to the reaction mixture. This solution is degassed by bubbling N₂ through it for 20 minutes. 0.81 mg AIBN (2,2'-azo-bis-isobutyronitrile) and 5 µL of a 5 mg/mL potassium persulfate solution are then added. 24 µL of inhibitor-free styrene is added and the mixture is stirred vigorously at 60°C for 24 hours. The resulting copolymer is washed several times with water and lyophilized. Assay by metachromatic toluidine blue dye-binding method of P.K. Smith et al. (Anal Biochem (1980) 109:466-473) gives an incorporation of 1.3 wt% heparin into the copolymer (designated, CH9-39).

Example 4

Copolymerization of Hydroxyethylmethacrylate

(HEMA) With Allyl Derivative of Heparin

Fragments From Heparinase Digestion

13.5 mg allyl glycoside of heparin (prepared as described in paragraph B of preparation A) is dissolved in 400 µL of methanol:water (1:1), and 100 µL of inhibitor-free HEMA is added to it. This mixture is degassed by bubbling N₂ through the solution for 20 minutes. One µL TEMED (N,N,N',N'-tetramethylethylenediamine) and 5 µL of a 100 mg/mL ammonium persulfate solution are then added and the reaction mixture is left at room temperature for 24 hours. The resulting jelly-like copolymer is alternately washed (following M.R. van de Mark et al., in Advances in Biomedical Polymers, E.A. Gebelein, ed., Polymer Science and Technology, Vol. 35, Plenum Press, New York, 1987; pp. 373-380) three times

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for 30 minutes each in pH 10.0 solution (1:1, isopropanol:H₂O) and a pH 3.0 solution (H₂O). After the final washing, the copolymer is lyophilized and assayed for heparin content which yields 0.1 wt% heparin present in the copolymer (designated, CH9-42).

Example 5

Copolymerization of Acrylonitrile With Heparin Fragments Produced by Enzymatic Digestion

44 mg of heparin disaccharide (prepared as described in part A of preparation A) is dissolved in 500 µL of water. 52 µL of inhibitor-free acrylonitrile is added and the mixture is made 1% in TEMED by adding 5.5 µL of the reagent. The solution is degassed by gently bubbling N₂ through it for 20 min. 5 µL of a 110 mg/mL ammonium persulfate in DMSO (dimethylsulfoxide) solution is added after degassing and the copolymerization mixture is warmed to 70°C while stirring vigorously. After 2 hours at 70°C, the mixture is removed from the water bath, the precipitate is centrifuged out, washed three times with water, and then dried (designated CH8-11P).

Example 6

Copolymerization of Toluene Diisocyanate and Ethylene Glycol With Heparin Fragments From Deaminative Cleavage With Nitrous Acid

A. Low molecular weight heparin (Sigma H5640, average molecular weight 4,000-6,000 Daltons) is reduced with sodium borohydride. 110 mg of the heparin is dissolved in 2 mL of water and a solution of 76 mg of NaBH₄ in 1 mL of ice-cooled water is added to it. Reduction of the anhydromannosyl moiety of heparin is carried out overnight (approximately 20 hrs) at room temperature. The reaction mixture is acidified with

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glacial acetic acid until the pH is stabilized to 5.2. The excess borohydride is removed by repeated evaporation (3 times) with methanol, and finally the reduced heparin is lyophilized.

B. 101 mg of reduced heparin (approximately, 17-25 mmole) is dissolved in 5 mL of formamide by warming up to 70°C. The solution is mixed with 100 µL of ethylene glycol (1.8 mmole) and diluted with 5 mL of dimethylsulfoxide. The mixture is cooled in an ice-bath, and 1 mL of tolylene-2,4-diisocyanate (7 mmole) which is in excess of the polyhydroxy components is added. The copolymerization reaction is continued for about 64 hrs with stirring while the mixture is gradually warmed to room temperature (20°C). The copolymer is isolated by pouring the resulting viscous mixture into chloroform. The precipitate is washed well with chloroform, ethanol, and finally with water until the supernatant is free from unreacted heparin, the latter being determined by the carbazole method for uronic acid. The washed copolymer is lyophilized and assayed for heparin by the dye-binding method (Smith et al., 1980) which shows that the product contains 0.7 wt% heparin. The copolymer (designated, CF3-22C) is insoluble in water but dissolves readily in dimethylsulfoxide, and the NMR spectra relates closely to aromatic residues in the polyurethane structure as well as some new bands assigned to heparin, as shown in Figure 5.

C. The same procedure as described in paragraph B above is followed, but a solution containing 93 mg of reduced heparin (approx. 19 mmole, prepared as in paragraph A) in 3 mL of formamide to which 46.5 µL of ethylene glycol (0.83 mmole) is added and further diluted with 3 mL of dimethylsulfoxide is prepared. This is followed by the addition of 121 µL of tolylene-2,4-diisocyanate (0.85 mmole, equimolar to the total f

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reduced heparin and ethylene glycol) to the solution, cooled in an ice-bath, and the reaction is left to proceed with stirring under N_2 atmosphere for 24 hours while the mixture gradually reaches room temperature ($21^\circ C$). The resulting copolymer (designated, CH9-21C) after appropriate washing, drying and assay shows heparin content of 1.97 wt%.

Example 7

Anticoagulant and Antithrombotic Activity of Some Copolymers Incorporating Heparin Fragments

Copolymers are evaluated for anticoagulant and antithrombotic activity by the APTT (Activated Partial Thromboplastin Time, Sigma Procedure No. A7668) and anti-Factor Xa (Sigma Technical Bulletin No. 870, Heparin in Plasma) assays, the latter being based on the neutralization of activated factor X (Xa) by its plasma inhibitor (antithrombin III). The ratio of anti-Xa to APTT is calculated from these results.

The ratio of anti-Xa activity to APTT is important in determining a substance's antithrombotic potential with respect to its anticoagulant activity. Substances with a high ratio appear to be more antithrombotic and carry with them less risk of hemorrhage. This is because the anti-Xa assay measures the ability of a substance to block the formation of thrombin at an early stage in the coagulation cascade, whereas the APTT measures the ability of a substance to stop coagulation at many steps along the coagulation sequence. Thus, anti-Xa activity specifically relates to the local antithrombotic potential, while APTT is a measure of the global anticoagulant activity. Commercial heparin has an anti-Xa/APTT ratio of approx. 1 and the low molecular weight heparin from Sigma has a ratio of approximately 2.

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Table I shows results from analyses of copolymers prepared as described in Examples 1 and 2. These copolymers are soluble in water, and can thus be assayed directly by the APTT and anti-Xa methods. Analyses of these copolymers prepared with small heparin fragments show that they generally retain the high anti-Xa/APTT ratios characteristic of the short oligosaccharides used to prepare the copolymers. For example, the anti-Xa/APTT ratios for heparin fragments of size octa-, deca- and dodecasaccharide by preparation A are 4.3, 7.4 and 6.5, respectively. Similarly, anti-Xa/APTT ratios for heparin fragments of size octa- and decasaccharide by preparation B are 10.4 and 10.0, respectively. One of the copolymers, namely the one designated CH8-36A, shows higher anti-Xa/APTT compared to the corresponding fragments, perhaps due to a variation in the inherent activity of the fragments resulting from different preparations or due to a better utilization of the active fragments which are randomly incorporated at a distance from each other and as pendant moieties into the copolymer backbone.

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Table I

Lot Number Designation	Copolymer Composition	Wt % Heparin	APTT (U/mg)	Anti-Xa (U Heparin/ mg)	Anti- Xa/APTT Ratio
CH6-131D	Heparin octa- saccharide: acrylamide by Example 1	13	7.7	12	1.6
CH6-131E	Heparin deca- saccharide: acrylamide by Example 1	26	9.2	35	3.8
CH6-131F	Heparin dodeca- saccharide: acrylamide by Example 1	39	11.0	55	5.0
CH8-23D	Low molecular weight heparin: acrylamide by Example 2	22	10.0	135	13.5
CH8-32B	Heparin octa- saccharide: acrylamide by Example 2	28	11.9	127	10.7
CH8-36A	Heparin deca- saccharide: acrylamide by Example 2	35	8.9	175	19.7

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Example 8Bead Copolymerization of Enzymatically-Cleaved
Heparin and Acrylamide

Fragments of heparin are prepared by digestion with heparinase from *F. heparinum* as described in preparation A (part A). In a typical copolymerization procedure, an aqueous monomer solution and an organic phase are prepared. The monomer phase contains 60 mg heparin fragments of size ranging from octasaccharide to hexadecasaccharide, 240 mg acrylamide, 60 mg polyvinylpyrrolidone, and 20 mg N,N¹-methylene-bis-acrylamide (Bis) dissolved in 1.6 mL type I water, while the organic phase consists of a mixture of 11 mL toluene, 5 mL chloroform and 80 µL Span 85. The monomer phase is cooled in an ice-bath to about 0°C and is mixed with 160 µL of a 50 mg/mL ammonium persulfate solution and 20 µL TEMED (N, N, N¹, N¹ - tetramethylethylenediamine). This solution is pipetted into the organic phase contained in a three-neck round-bottomed flask which is being stirred rapidly with a paddle-stirrer. Nitrogen gas is gently blown through the flask until all of the monomer phase has been added. The mixture is allowed to react for 30 min at 25°C while stirring at high speed and then at 65°C for 60 min at low speed. The reaction mixture is transferred to a sintered glass funnel and washed with type I water, acetone and finally methanol. The copolymer beads are suspended in water and allowed to soak for five days to remove the polyvinylpyrrolidone and any unreacted components. The beads are washed again on a sintered glass funnel with a large volume of type I water and methanol. The solid copolymer is then vacuum-dried at 60°C for 4-5 h. This is assayed for heparin content using the toluidine blue dye binding method of Smith et al. (1980), indicating a content of 2 mg of heparin/g of solid beads (0.2% heparin by weight).

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Example 9Copolymerization of Aminoethylmethacrylate-Derivatized
Heparin with Acrylamide Using bis-acrylamide as a
Cross-linker

- A. Low molecular weight heparin (4,000 - 6,000 Da), obtained from Sigma Chemical Co., is derivatized with aminoethylmethacrylate (AEM) following the procedure described in Example 2 (paragraph A) and also in Example 3. The heparin derivative is purified further by dialysis in 3,500 MWCO Spectrapore tubing (to remove excess of aminoethylmethacrylate) and the inner dialysate is lyophilized.
- B. A typical copolymerization mix contains 30 mg acrylamide, 30 mg AEM-derivatized low molecular weight heparin (AEM-LMWH) and 2.5 mg bis-acrylamide, dissolved in 1.0 mL type I water and cooled to about 0°C in an ice-bath. To this is added 160 µL of a 50 mg/mL ammonium persulfate solution and 20 µL TEMED. The solution is mixed well and nitrogen gas is gently bubbled through the reaction mixture. The mixture is left to polymerize at room temperature overnight. The reaction mixture is dialyzed in 3,500 MWCO tubing against 50 mM EDTA and subsequently against type I water. The inner dialysate is lyophilized and filtered through a column of Sephadex G-150 superfine gel using 0.2 M NaCl as the running buffer. Fractions (15 min) are collected at a flow rate of approximately 0.1 mL/min and assayed for uronic acid to identify the fractions containing copolymer. These fractions are pooled, desalted and lyophilized. Uronic acid,

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APTT and anti-Factor Xa assays are performed on the copolymer to determine the heparin content and biological activity. Results show a heparin content of 17%, APTT activity of 2.9 U/mg and anti-Xa activity of 6.63 U/mg of copolymer.

Example 10

Copolymerization of Allyl-heparin with Acrylamide Using bis-acrylamide as a Cross-linker

Allyl glycoside of heparin fragment obtained from enzymatic digestion is prepared as described in preparation A (paragraph B). The copolymerization follows essentially the same procedure as in Example 9 except that 30 mg of allyl-heparin (hexadecasaccharide) is used instead of AEM-heparin. The copolymer, appropriately purified and lyophilized, has a heparin content of 11% and an APTT activity of only 0.62 U/mg of copolymer, while there is practically no anti-Xa activity.

Example 11

Copolymerization of Allyl-heparin with Acrylonitrile

Enzymatically produced heparin hexadecasaccharide is derivatized to allyl glycoside as before. 20 mg of allyl-heparin is dissolved in 200 μ L of inhibitor-free acrylonitrile and mixed with 6 mg of AIBN (2,2¹ - azo-bis-isobutyronitrile) in 150 μ L of type I water. The mixture is stirred at 70°C in an oil-bath for 4.5 h. A solid copolymer is formed which is recovered by centrifugation and washed extensively with type I water. The copolymer is dried and assayed for heparin content using the toluidine blue dye binding method. The biological activity is determined by the APTT and anti-Factor Xa assays. The results of these assays indicate a

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heparin content of 8%, while the APTT and anti-Xa activities are 0.15 U/mg and 1.0 U/mg of solid, respectively. A typical NMR spectrum of the copolymer is given in Figure 6. The spectrum clearly shows the absence of the characteristic signal corresponding to the allyl group.

Example 12

Copolymerization of AEM-derivatized Heparin with Acrylonitrile

AEM-heparin, prepared as described in Example 9 (paragraph A), is dissolved in water. 25 mL of AEM-heparin solution (10 mg/mL) is warmed in a 100 mL round-bottomed flask to 70°C in a thermostated oil-bath. A solution of 1.7 mg AIBN in 5 mL of inhibitor-free acrylonitrile is added to the flask and the temperature is raised to 75°C. The mixture is allowed to react for 3 h with stirring, whereupon a white precipitate is formed inside the flask. The precipitate is removed and washed extensively with type I water in a sintered glass funnel. This is resuspended in type I water and lyophilized. Heparin content is found to be 0.5%, and the copolymer shows high anticoagulant activity.

Example 13

Copolymerization of Aminoethylmethacrylate with Acrylonitrile and Derivatization of Copolymer with Low Molecular Weight Heparin

2.5 g of AEM is dissolved in 10 mL of dimethyl sulfoxide (DMSO) and to the solution are added 1 mL of inhibitor-free acrylonitrile (1:1 molar ratio of AEM to acrylonitrile) and 0.3 g of AIBN. The solution is warmed to 70°C while stirring and allowed to react for 4 h. The reaction mixture turns into a viscous solution and the copolymer is precipitated by dropwise addition of the

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reaction mixture to 1 L of methylene chloride with vigorous stirring. The precipitated copolymer is filtered in a sintered glass funnel and washed well with methylene chloride. The copolymer is dried in a vacuum oven at 70°C and crushed into a powder.

250 mg of the AEM-acrylonitrile copolymer is dissolved in 14 mL of 5% phenol and to this is added 63 mg of low molecular weight heparin (4,000 - 6,000 Da) dissolved also in 1 mL of 5% phenol. The mixture is stirred gently for 24 h at room temperature. 10 mg of sodium cyanoborohydride is then added (pH - 5) and the reaction is continued for another 48 h. The derivatized copolymer is dialyzed against type I water in a 6,000 - 8,000 MWCO Spectrapore tubing and lyophilized. Heparin content is determined to be 5.5% using the uronic acid assay. Biological activity is measured by the APTT and anti-Factor Xa assays which give 8.8 U/mg and 16.7 U/mg, respectively.

Example 14

Copolymerization of Aminoethylmethacrylate with 2-hydroxyethyl Methacrylate (HEMA) Followed by Derivatization with Low Molecular Weight Heparin

2.56 g of AEM and 2 mL of HEMA are dissolved in 28.5 mL of DMSO in a 100 mL round-bottomed flask to which is added 855 mg of VAZO-67 (2, 2¹ - azo-bis-methylbutyronitrile). The flask is fitted with a reflux condenser and placed in an oil-bath heated to 70°C. The mixture is allowed to react for 4 h at this temperature. The copolymer is precipitated by adding the reaction mixture dropwise to 500 mL of dichloromethane while stirring vigorously and then filtered in a sintered glass funnel. The copolymer is redissolved in DMSO, r precipitated in dichloromethane and filtered again.

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This procedure is repeated once more and the copolymer is dried in a vacuum oven at 60°C.

100 mg of the AEM-HEMA copolymer and 25 mg of low molecular weight heparin (4,000 - 6,000 Da) are dissolved in 9 mL of type I water and mixed gently overnight at room temperature. 10 mg of sodium cyanoborohydride is added to the solution, the latter being slightly acidic (pH - 6), and stirring continued overnight at room temperature. The reaction mixture is dialyzed in 6,000 - 8,000 MWCO tubing against type I water and the inner dialysate is lyophilized. Uronic acid assay shows a heparin content of 5.1%, while the APTT and anti-Xa activities are 17.6 and 88 U/mg, respectively.

Example 15

Copolymerization of Aminoethylmethacrylate with 4-styrenesulfonic Acid Followed by Derivatization with Low Molecular Weight Heparin

0.5 g of AEM and 0.62 g of 4-styrene sulfonic acid are dissolved in 25 mL of type I water. 0.75 g of VAZO-67 is dissolved in the mixture to give a solution containing 3% initiator. The solution is warmed to 70°C and is allowed to react for 4 h at this temperature with stirring. A yellow precipitate is obtained and separated from the reaction mixture. This is redissolved in 50 mL of 0.1 M sodium phosphate buffer (pH = 10.9), dialyzed against type I water in a 3,500 MWCO tubing, and the copolymer is lyophilized.

40 mg of the AEM-styrene sulfonic acid copolymer and 10 mg of low molecular weight heparin (4,000 - 6,000 Da) are dissolved in 3.5 mL of type I water and stirred slowly for 24 h at room temperature. 10 mg of sodium cyanoborohydride is then dissolved in the slightly acidic solution and mixed at room temperature for another

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24 h. The mixture is dialyzed in 6,000 - 8,000 MWCO tubing against type I water (to remove unbound heparin) and the inner dialysate is lyophilized. The derivatized copolymer is found to have a heparin content of 22% by the uronic acid assay. It shows an APTT and an anti-Xa activity of 9.8 and 140 U/mg, respectively.

Example 16

Copolymerization of an Aminoethylmethacrylate Derivative of Heparin with 4-styrenesulfonic Acid

The AEM derivative of low molecular weight heparin (4,000 - 6,000 Da) is prepared as noted in Example 9 (paragraph A). 400 mg of 4-styrenesulfonic acid is dissolved in 10 mL of an AEM-heparin solution in water (10 mg/mL). 300 mg of VAZO-67 is added and the temperature is raised to 70°C. The polymerization reaction is carried out overnight with stirring. The solution is then dialyzed in a 6,000 - 8,000 MWCO tubing against 50 mM EDTA followed by type I water, and lyophilized. Heparin content of the dried copolymer is found to be 12.7% by the uronic acid assay. Biological activity is determined using the APTT and anti-Factor Xa methods, which give 16 U/mg and 160 U/mg, respectively.

Example 17

Copolymerization of Aminoethylmethacrylate with Vinyl Acetate and Derivatization of the Copolymer with Low Molecular Weight Heparin

Vinyl acetate (1.75 g) and AEM (0.25 g) are dissolved in DMF (25 mL) and VAZO-67 (20 mg) is added to the solution under a nitrogen atmosphere. The solution is heated at 70°C in a constant temperature oil-bath for 20 h. The resulting solution is concentrated by distilling under vacuum and the copolymer is precipitated by suspending the viscous solution in water with vigorous

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stirring. The precipitate is filtered, washed repeatedly with water, and lyophilized. The copolymer is derivatized with LMWH (Sigma) in DMF solution following the same general procedure as described in Examples 13-15. This shows a heparin content of 0.26% (by weight).

Example 18

Copolymerization of Tolylene Diisocyanate and Low Molecular Weight Heparin (LMWH) Derivatized with Tris-(hydroxymethyl) Aminomethane

- A. Low molecular weight heparin (4,000 - 6,000 Da), produced by nitrous acid depolymerization, is obtained from Sigma Chemical Co., USA. This is pre-derivatized with a 20-25 times molar excess of Tris in a formamide solution by reductive amination in the presence of sodium borohydride. Typically, 50 mg Tris, 101.2 mg LMWH and 5.5 mg NaBH_4 are dissolved in 5 mL (total volume) formamide, and the solution is left overnight (16-22 h) at room temperature. The mixture is transferred into a small round-bottomed flask set up in an ice-bath, fitted with rubber septum, and nitrogen gas is bubbled through it. Approximately 15 min later, 1 mL tolylene-2,4-diisocyanate (large excess) is added from a syringe, and the solution is diluted with 5 mL dimethylsulfoxide. The copolymerization is continued overnight (about 18 h) with stirring under the N_2 atmosphere at 4°C. The resulting copolymer is precipitated by adding a mixture of methanol and chloroform (100 mL each). The precipitate is filtered and washed extensively with chloroform, methanol, methanol-water and finally pure water. The copolymer is dried in a vacuum oven at 70°C,

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and it shows a heparin content of 5.24 $\mu\text{g}/\text{mg}$ of solid by the toluidine blue dye binding method of Smith et al. (1980). The biological activity is measured by the APTT and anti-Factor Xa assays which give 7.3 mU/mg and 8.35 mU/mg of solid, respectively. A typical FTIR spectrum is given in Figure 7 which shows strong absorption bands for hydroxyl groups at 3400 cm^{-1} and urethane linkages at 1700 cm^{-1} .

- B. The same procedure as described in paragraph A above is followed, except that 114 mg LMWH, 121.4 mg Tris and 12.6 mg NaBH_4 are dissolved in a mixture of 2 mL dimethylformamide (DMF) and 6 mL formamide for the pre-derivatization of LMWH. The copolymerization is carried out exactly under the same conditions as above, but in presence of 100 μL dibutyltin dilaurate used as a catalyst and with 145 μL tolylene-2,4-diisocyanate (equimolar to the amount of Tris). The reaction is continued for 5 h in an ice-bath and then at room temperature for another 16 h. The copolymer is precipitated with chloroform, washed well with methanol and water, and dried in a vacuum oven at 60°C . This yields 0.4 μg heparin/mg of solid with APTT and anti-Xa activities of 13 mU/mg and 14 mU/mg, respectively.

Example 19

Copolymerization of an Isocyanate-Terminated Prepolymer with Tris-(hydroxymethyl) Aminomethane-Derivatized Low Molecular Weight Heparin

The same procedure as described in Example 18 is followed with the exception that 81 mg LMWH, 49.6 mg Tris and 5.1 mg NaBH_4 are dissolved in 3 mL formamide.

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The derivatization of LMWH with Tris is performed overnight (19 h) at room temperature. This is followed by reaction with 2.04 g Hypol 2000 (an isocyanate-terminated prepolymer containing 1.6 mg NCO/g), diluted with 7 mL dimethylsulfoxide and transferred into a round-bottomed flask under N_2 atmosphere. The reaction is continued for about 22 h at 4°C with the addition of 18 mL chloroform after 6 h to reduce the viscosity of the mixture. Finally, a semi-solid mass is obtained which is dispersed in 200 mL $CHCl_3$ and then precipitated from a large volume (~800 mL) of ethanol. The precipitate, washed extensively with methanol and water, is dried in a vacuum oven as before. The copolymer has a heparin content of 1.05 μ g/mg of solid, APTT activity of 8.4 mU/mg and anti-Xa activity of 7.2 mU/mg.

Example 20

Copolymerization of Tolylene Diisocyanate with Serinol Hydrochloride and Derivatization of the Polymer with Low Molecular Weight Heparin

One mmole serinol hydrochloride (127.4 mg) is dissolved in 5 mL dimethylacetamide to which is added 142 μ L tolylene-2,4-diisocyanate (equimolar) and 50 μ L dibutyltin dilaurate (catalyst). The mixture is allowed to react in a rotary evaporator at 65°C for approximately 30 min and then at room temperature (21°C) for 18 h. The copolymer is precipitated by suspending the mixture in 200 mL chloroform, washed well with methanol and water, filtered, and dried in a vacuum oven. 35.6 mg of the dried copolymer is dissolved in formamide, and the solution mixed with 61.3 mg LMWH and 8.4 mg $NaBH_4$, also in formamide, for a total of 4 mL. The mixture is left overnight (~ 17 h) at room temperature for derivatization of the copolymer, followed by precipitation and washing with water. The precipitate is vacuum-dried and shows

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0.26 μg of heparin/mg of solid by the toluidine blue dye binding method, while the APTT and anti-Xa activities are 30 mU/mg and 240 mU/mg, respectively.

Example 21

Copolymerization of Tolylene Diisocyanate with Tris-(hydroxymethyl) Aminomethane and Derivatization of the Copolymer with Low Molecular Weight Heparin

The same procedure as described in Example 20 is used except that 120 mg Tris (1 mmole) is dissolved in 6 mL dimethylformamide. An equimolar amount of tolylene-2,4-diisocyanate (142 μL) is added and the copolymerization is carried out in presence of 50 μL dibutyltin dilaurate. The copolymer is precipitated, washed, and dried in a vacuum oven as before. The dried copolymer (36 mg) is dissolved in formamide (4 mL) together with low molecular weight heparin (61.3 mg) and sodium borohydride (8.4 mg). The derivatization of the copolymer is continued overnight at room temperature. The resulting product is dialyzed extensively against pure water using 6,000 - 8,000 MWCO Spectrapore dialysis tubing. The dialyzed solution is concentrated in a Rotovap and then dried in a vacuum oven at 70°C. The product contains 64 μg of heparin/mg of solid and shows high anti-Xa activity.

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We claim:

1. A biologically compatible copolymer suitable for the construction or coating of biomedical devices which contact blood, which copolymer is antithrombotic and nonthrombogenic, said copolymer comprising:

at least one glycosaminoglycan fragment monomeric unit copolymerized with at least one non-glycosaminoglycan monomeric unit.

2. The copolymer of claim 1 wherein the glycosaminoglycan fragment is prepared as an enzymatic degradation product or as a chemical degradation product of a high MW glycosaminoglycan, and wherein said glycosaminoglycan fragment is antithrombotic and nonthrombogenic.

3. The copolymer of claim 2 wherein said glycosaminoglycan fragment is derived from heparin, dermatan sulfate, chondroitin sulfate Type A, chondroitin sulfate Type C or hyaluronic acid.

4. The copolymer of claim 3 wherein the glycosaminoglycan fragment is derived from heparin.

5. The copolymer of claim 4 wherein the glycosaminoglycan fragment is obtained by depolymerization of heparin and recovering size fragments ranging from penta- to hexadecasaccharides.

6. The copolymer of claim 5 wherein said depolymerization is effected by heparinase digestion, nitrous acid treatment or periodate cleavage.

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7. The copolymer of claim 1 wherein the nonglycosaminoglycan monomeric unit is an unsaturated monomer or an oligomer thereof.

8. The copolymer of claim 7 wherein at least one said unsaturated monomer is of the formula $\text{CH}_2=\text{CXY}$ wherein X is H or CH_3 and Y is selected from the group consisting of $-\text{COOR}$, $-\text{CONH}_2$, $-\text{CONHR}$, $-\text{CONR}_2$, $-\text{CONH}(\text{CH}_2)_n\text{OH}$, $-\text{CONH}(\text{CH}_2)_n\text{OR}$, $-\text{COO}(\text{CH}_2)_n\text{OH}$, $-\text{COO}(\text{CH}_2)_n\text{OR}$, $-\text{OCOR}$, $-\text{OR}$, $-\text{Cl}$, $-\text{CN}$, $-\text{C}_6\text{H}_5$, substituted $-\text{C}_6\text{H}_5$, $-\text{OCO}(\text{CH}_2)_n\text{OH}$, $-\text{OCO}(\text{CH}_2)_n\text{OR}$, $-\text{OCO}(\text{CH}_2)_n\text{NH}_2$, $-\text{OCO}(\text{CH}_2)_n\text{NHR}$, and $-\text{OCO}(\text{CH}_2)_n\text{NR}_2$, wherein n is an integer of 1-4 and R is a straight or branched chain alkyl group of 1-4C.

9. The copolymer of claim 8 wherein said unsaturated monomer is acrylamide, styrene, 4-styrene sulfonic acid hydroxyethyl methacrylate, acrylonitrile, or aminoethylmethacrylate.

10. The copolymer of claim 7 wherein the unsaturated monomer is a diisocyanate or an oligomer of said diisocyanate with a diol.

11. The copolymer of claim 1 wherein said glycosaminoglycan fragment is a low molecular weight heparin derivatized at the nonreducing terminus.

12. The copolymer of claim 11 wherein the heparin derivative is the allyl derivative or the aminoethylmethacrylate derivative.

13. The copolymer of claim 1 which further includes crosslinking.

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14. The copolymer of claim 13 wherein said crosslinking is provided by a diunsaturated crosslinker.

15. The copolymer of claim 1 wherein said glycosaminoglycan fragment monomeric unit is supplied subsequent to polymerization of the nonglycosaminoglycan monomer components.

16. The copolymer of claim 1 wherein the copolymer is formed from a multiplicity of different glycosaminoglycan monomeric units.

17. The copolymer of claim 1 wherein the copolymer is formed from a multiplicity of different non-glycosaminoglycan monomeric units.

18. A medical device suitable for uses involving contact with blood which is constructed or coated using the copolymer of claim 1.

19. A method to prepare a biologically compatible copolymer suitable for the construction or coating of biomedical devices which contact blood, which copolymer is antithrombotic and nonthrombogenic which method comprises reacting at least one glycoaminoglycan fragment with at least one non-glycosaminoglycan component under conditions which effect copolymerization.

20. The method of claim 19 wherein said reacting is conducted in the presence of at least one crosslinking agent.

21. The method of claim 19 wherein the glycosaminoglycan fragment is prepared as an enzymatic degradation product or as a chemical degradation product

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of a high MW glycosaminoglycan, and wherein said glycosaminoglycan fragment is antithrombotic and nonthrombogenic.

22. The method of claim 19 wherein the glycosaminoglycan fragment is obtained by depolymerization of heparin and recovering size fragments ranging from penta- to hexadecasaccharides.

23. The method of claim 19 wherein the nonglycosaminoglycan monomeric unit is an unsaturated monomer or an oligomer thereof.

24. The method of claim 23 wherein said unsaturated monomer is of the formula $\text{CH}_2=\text{CXY}$ wherein X is H or CH_3 and Y is selected from the group consisting of $-\text{COOR}$, $-\text{CONH}_2$, $-\text{CONHR}$, $-\text{CONR}_2$, $-\text{CONH}(\text{CH}_2)_n\text{OH}$, $-\text{CONH}(\text{CH}_2)_n\text{OR}$, $-\text{COO}(\text{CH}_2)_n\text{OH}$, $-\text{COO}(\text{CH}_2)_n\text{OR}$, $-\text{OCOR}$, $-\text{OR}$, $-\text{Cl}$, $-\text{CN}$, $-\text{C}_6\text{H}_5$, substituted $-\text{C}_6\text{H}_5$, $-\text{OCO}(\text{CH}_2)_n\text{OH}$, $-\text{OCO}(\text{CH}_2)_n\text{OR}$, $-\text{OCO}(\text{CH}_2)_n\text{NH}_2$, $-\text{OCO}(\text{CH}_2)_n\text{NHR}$, and $-\text{OCO}(\text{CH}_2)_n\text{NR}_2$, wherein n is an integer of 1-4 and R is a straight or branched chain alkyl group of 1-4C.

25. A method to prepare a biologically compatible copolymer suitable for the construction or coating of biomedical devices which contact blood, which copolymer is antithrombotic and nonthrombogenic which method comprises reacting at least one glycoaminoglycan which is of low molecular weight with a prepolymerized nonglycosaminoglycan prepolymer under conditions which effect derivatization of said glycosaminoglycan to said prepolymer through the reducing terminus of said glycosaminoglycan.

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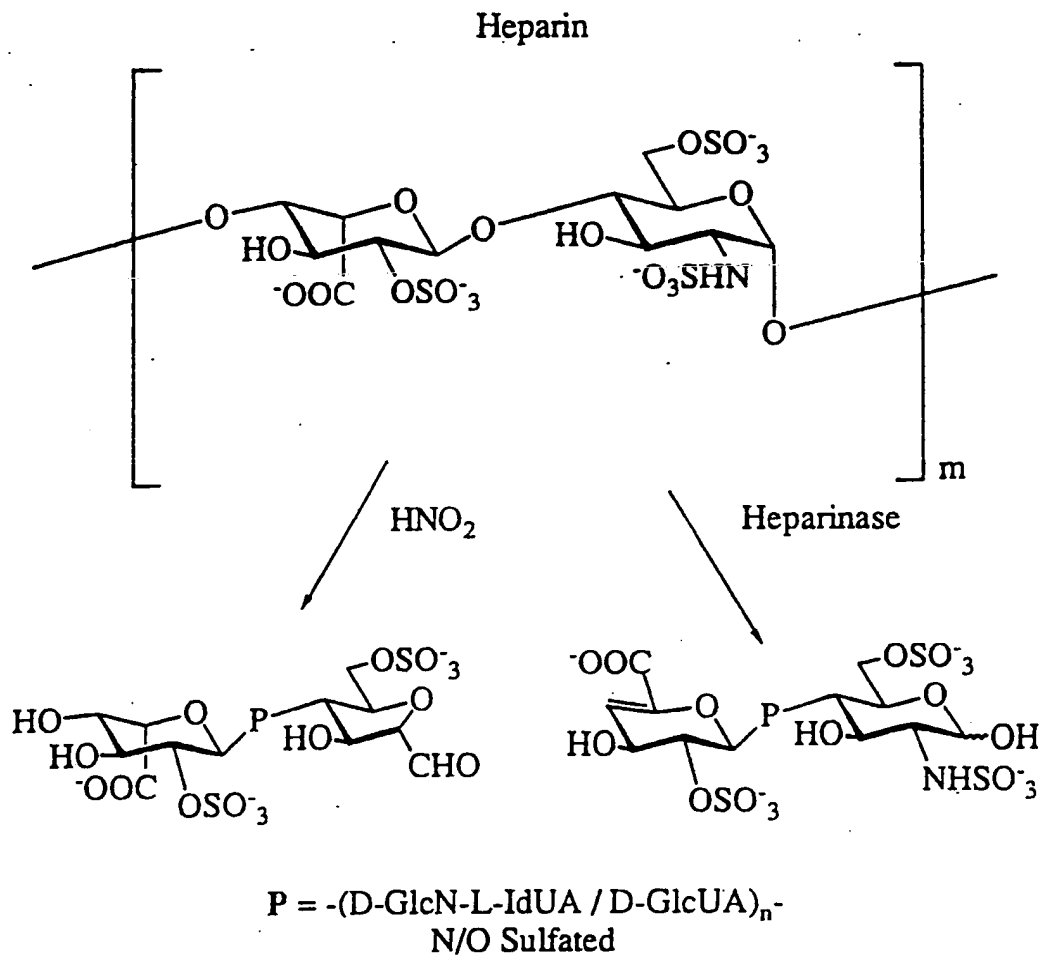
26. A water-soluble copolymer useful as a pharmacological agent, which copolymer is antithrombotic and nonthrombogenic, said copolymer comprising:

at least one glycosaminoglycan fragment monomeric unit copolymerized with at least one non-glycosaminoglycan monomeric unit.

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**N/O-Sulfated
(L-IdUA / D-GlcUA-D-GlcN)_m**



Uronosyl-2,5-Anhydro-D-Mannose-6-Sulfate

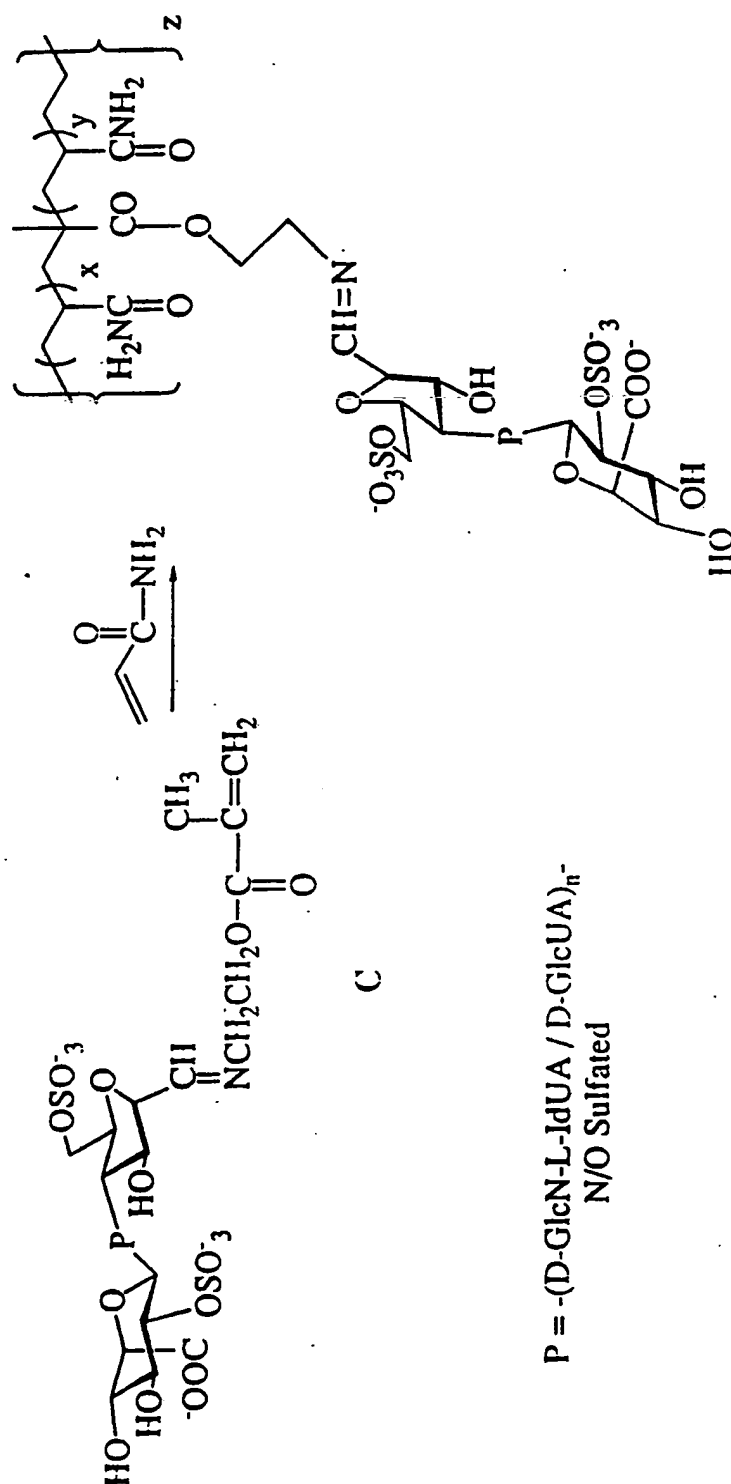
4,5-D-Uronate

A**B**

**Figure 1: Degradation of heparin and typical products from
(A) nitrous acid cleavage and (B) heparinase digestion**

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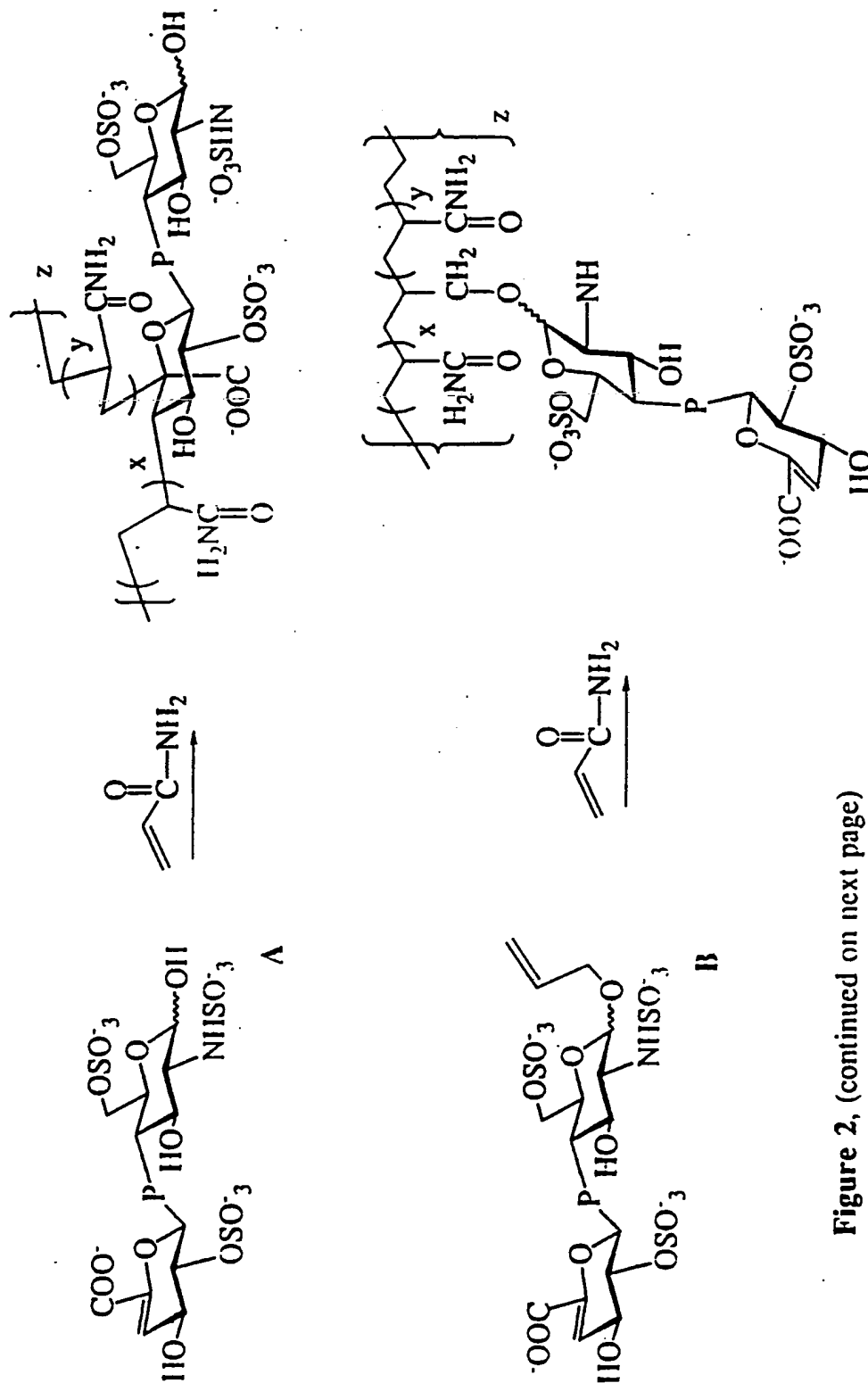
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$\text{P} = -(\text{D-GlcN-L-IdUA} / \text{D-GlcUA})_n-$
N/O Sulfated

Figure 2: Copolymerization scheme of acrylamide with typical (A) heparin fragment generated by heparinase digestion (Example 1), (B) allyl glycoside derivative of product from heparinase digestion, and (C) aminoethylmethacrylate derivative of nitrous acid cleavage product (Example 2).

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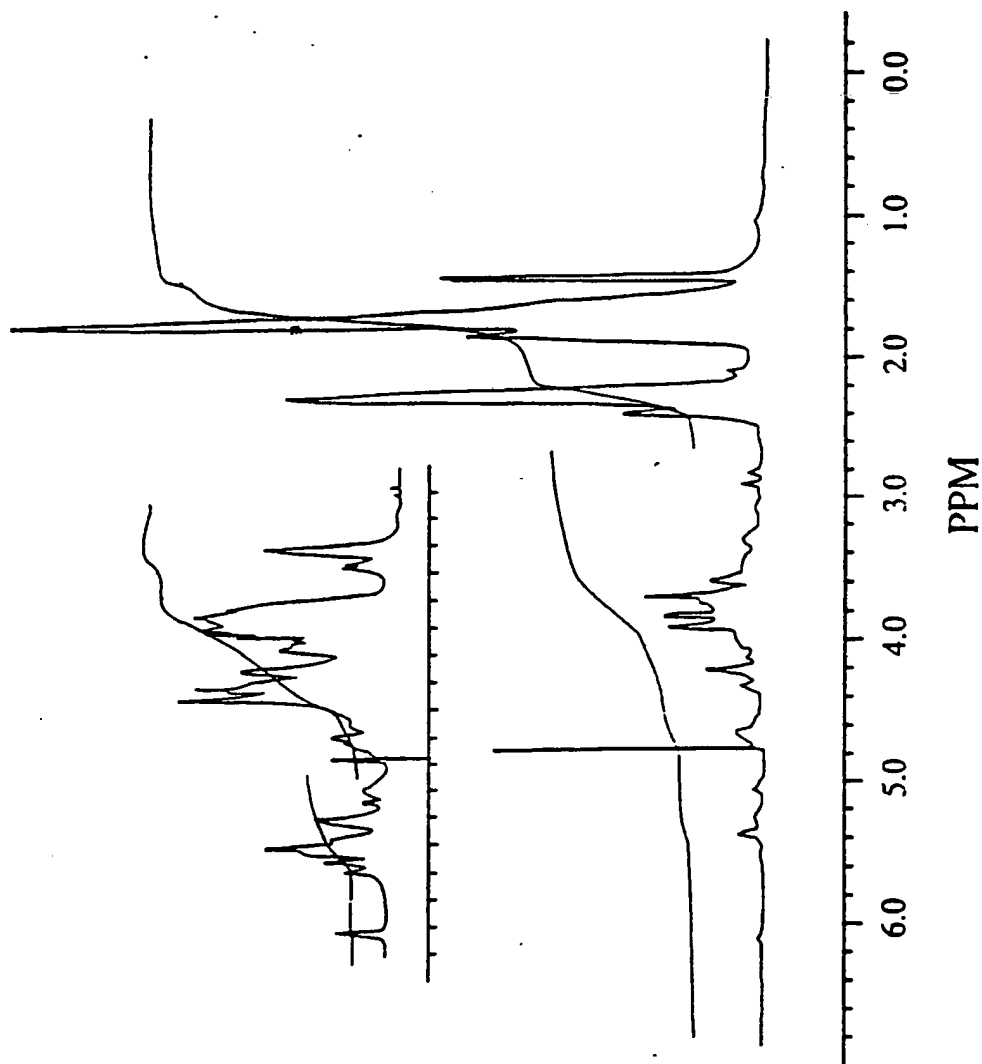


Figure 3: Proton NMR spectra in D_2O of a "putative copolymer" of acrylamide with heparin decasaccharide obtained from heparinase digestion (Preparation A). The copolymer (designated CH5-139, prepared by Example 1) contains 7.9 weight % heparin fragment after purification by desalting on a Sephadex G-25 column, followed by dialysis against 50 mM EDTA and finally against Type I water. Inset shows proton NMR spectra of corresponding heparin decasaccharide.

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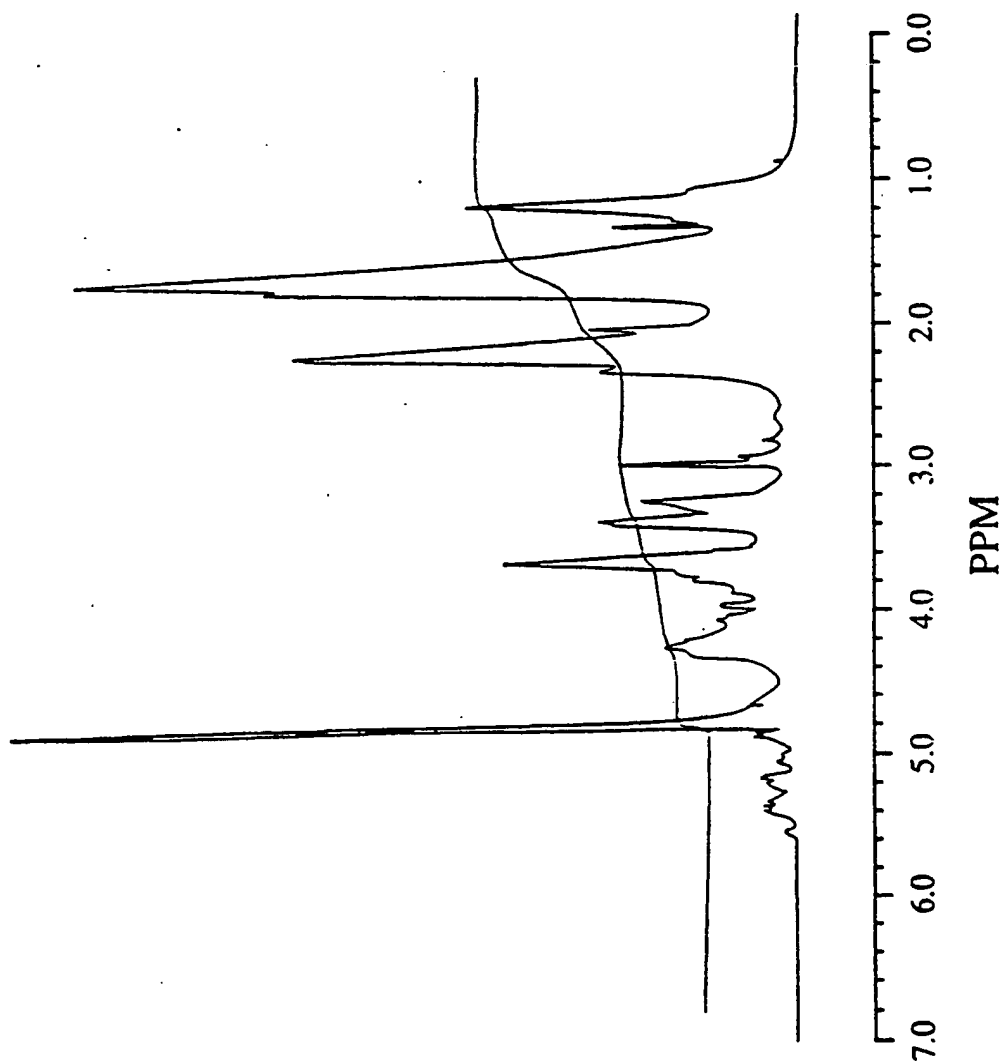


Figure 4: Typical proton NMR spectra in D_2O of a copolymer of acrylamide with aminoethylmethacrylate derivative of heparin octasaccharide produced by limited deaminative cleavage with nitrous acid (Preparation B). The copolymer (C118-32B, prepared by Example 2) contains 28 wt % heparin fragment, following purification on a Sepharose 4B column eluted with 0.2 M NaCl and dialysis against pure water.

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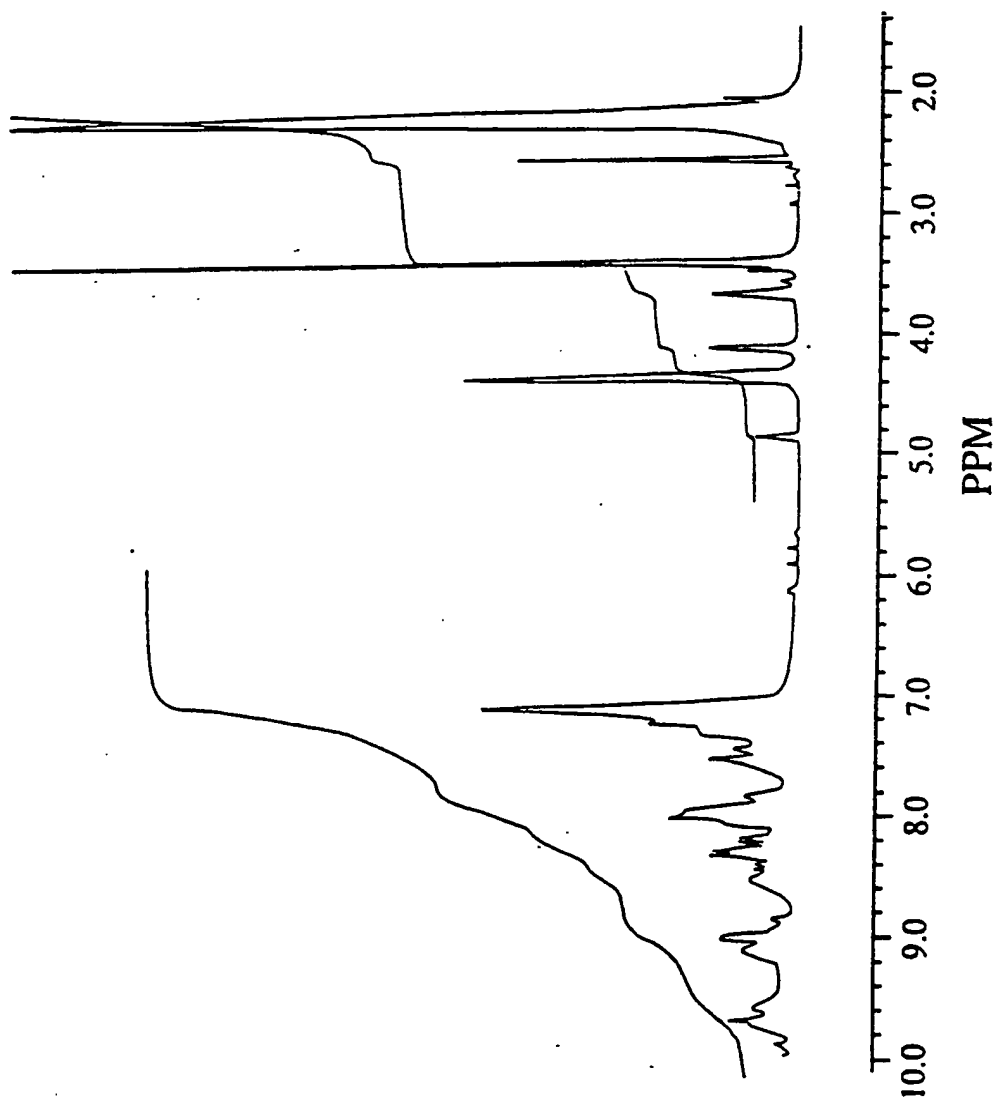
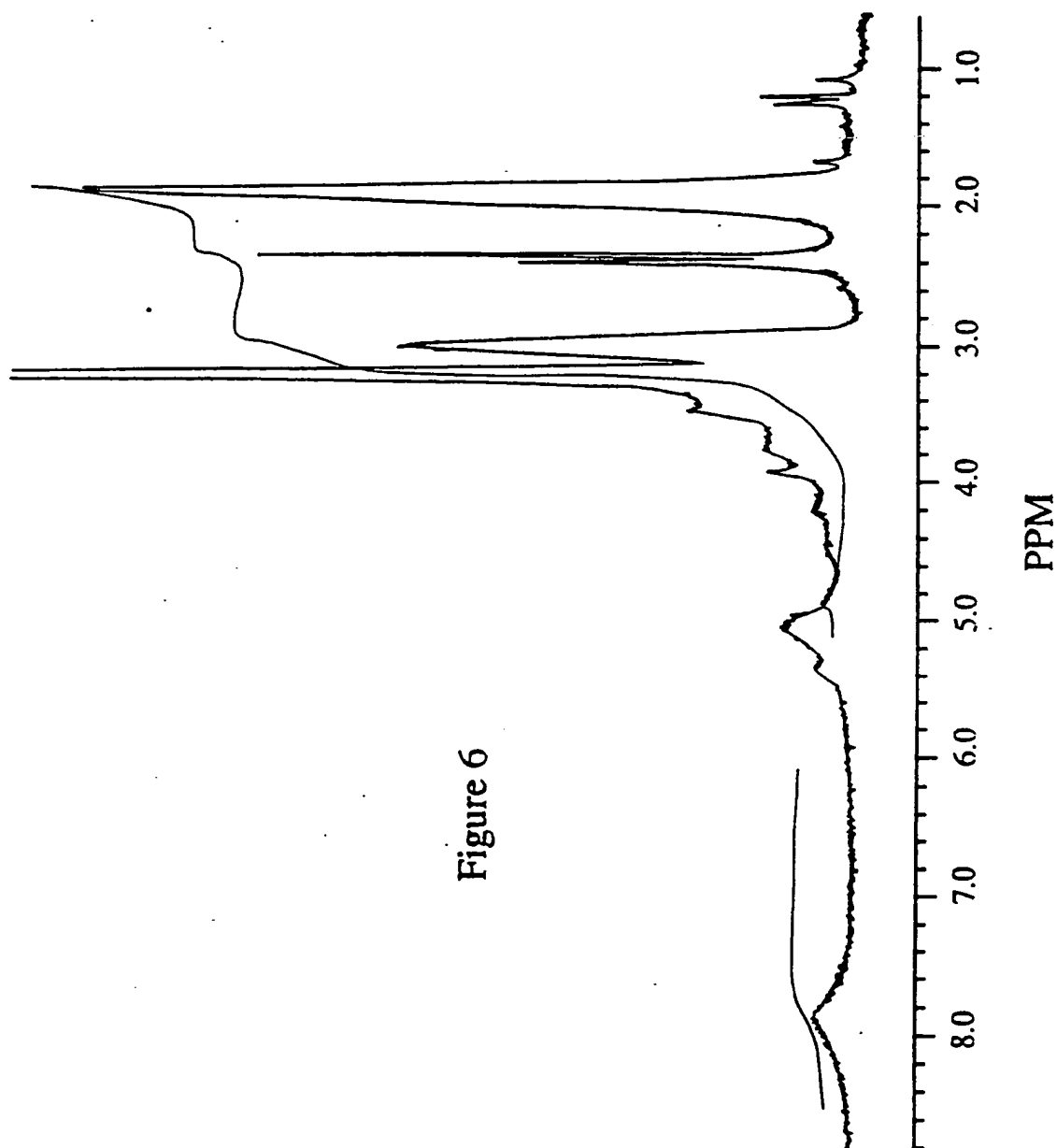
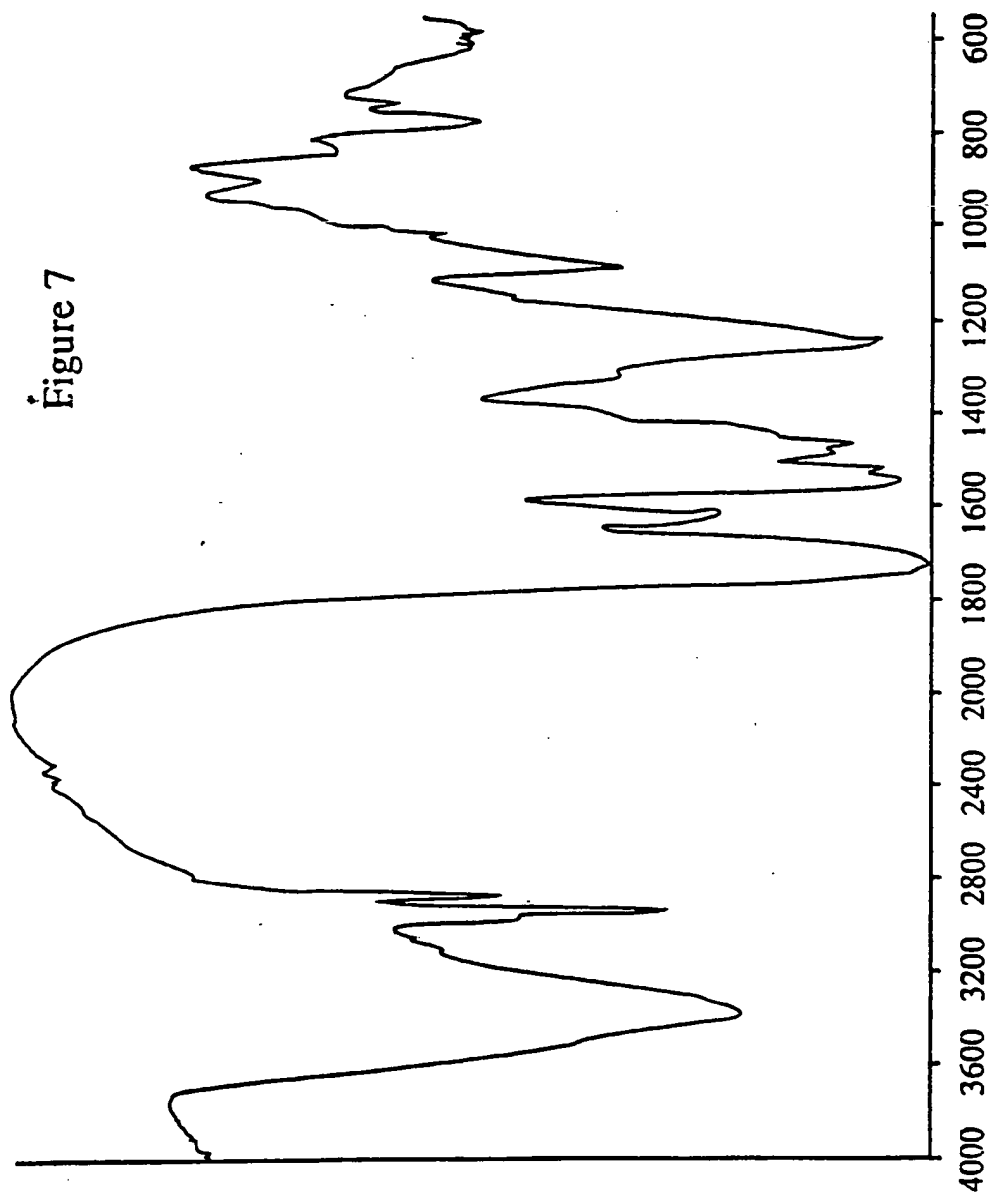


Figure 5: Proton NMR spectra in deuterated DMSO of a polyurethane-type polymer prepared by copolymerization of tolylene diisocyanate and ethylene glycol with low molecular weight heparin produced from nitrous acid cleavage. The copolymer (CF3-22C, prepared by Example 6) contains 0.7 wt % heparin after being extensively washed with chloroform, ethanol and finally with water until free from unreacted heparin and other monomers.

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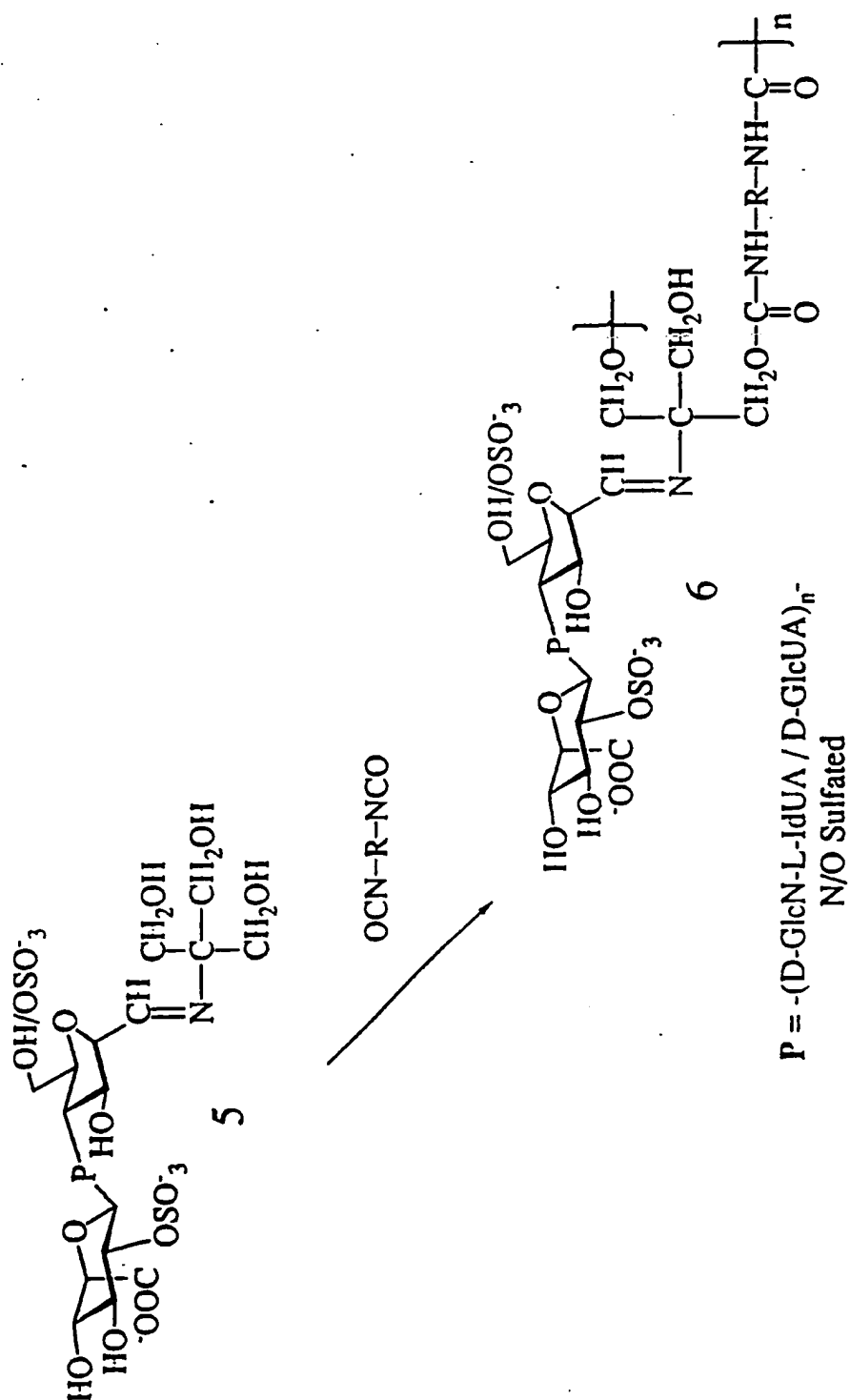
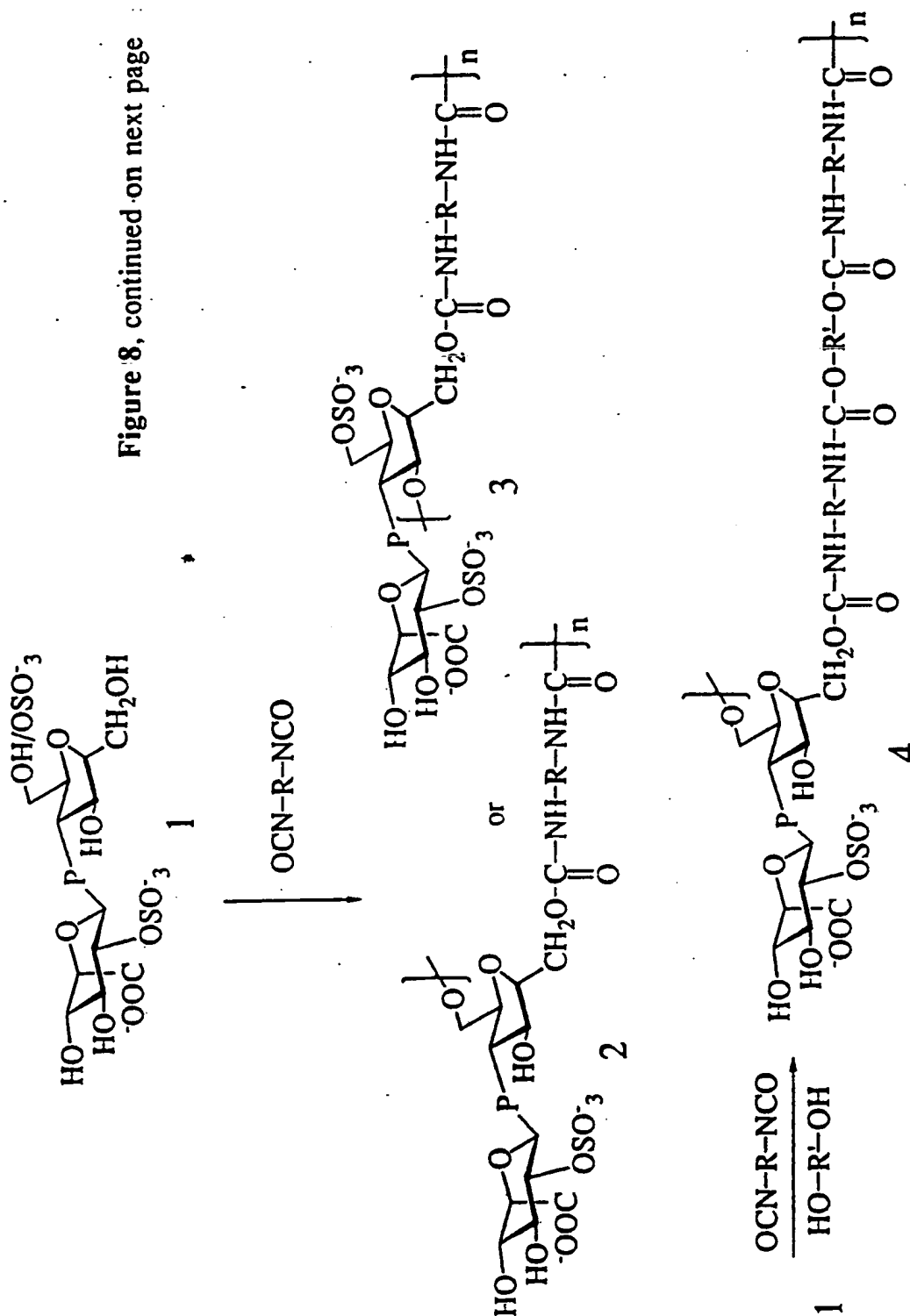


Figure 8: Copolymerization schemes for the synthesis of copolyurethanes with low molecular weight heparin fragments

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Figure 8, continued on next page



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INTERNATIONAL SEARCH REPORT

PCT/CA 91/00120

International Application No

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁴ According to International Patent Classification (IPC) or to both National Classification and IPC IPC ⁵ : A 61 L 33/00																	
II. FIELDS SEARCHED <div style="text-align: right; font-size: small;">Minimum Documentation Searched ⁷</div> <table style="width: 100%; border: none;"> <tr> <td style="width: 20%; border: none;">Classification System </td> <td style="border: none;">Classification Symbols</td> </tr> <tr> <td style="border: none;">IPC⁵ </td> <td style="border: none;">A 61 L, C 08 B, C 08 G, C 08 F</td> </tr> </table> <div style="text-align: center; font-size: x-small; margin-top: 5px;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸</div>			Classification System	Classification Symbols	IPC ⁵	A 61 L, C 08 B, C 08 G, C 08 F											
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IPC ⁵	A 61 L, C 08 B, C 08 G, C 08 F																
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹ <table border="1" style="width: 100%; border-collapse: collapse; font-size: x-small;"> <thead> <tr> <th style="width: 10%;">Category ¹⁰</th> <th style="width: 60%;">Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²</th> <th style="width: 30%;">Relevant to Claim No. ¹³</th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td style="vertical-align: top;">WO, A, 8700060 (BATTELLE MEMORIAL INS.) 15 January 1987 see the whole document --</td> <td style="text-align: center; vertical-align: top;">1-26</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td style="vertical-align: top;">US, A, 4521564 (SOLOMON et al.) 4 June 1985 see the whole document --</td> <td style="text-align: center; vertical-align: top;">1-26</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">Y</td> <td style="vertical-align: top;">American Chemical Society Symp. Ser., Carbohydrate sulfates, 8 May 1978, L. Mester et al.: "Heparin Derivatives of high molecular weight", pages 113-120 see the whole document --</td> <td style="text-align: center; vertical-align: top;">1-26</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">A</td> <td style="vertical-align: top;">US, A, 4239664 (TENG et al.) 16 December 1980 -- ./.</td> <td></td> </tr> </tbody> </table>			Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³	Y	WO, A, 8700060 (BATTELLE MEMORIAL INS.) 15 January 1987 see the whole document --	1-26	Y	US, A, 4521564 (SOLOMON et al.) 4 June 1985 see the whole document --	1-26	Y	American Chemical Society Symp. Ser., Carbohydrate sulfates, 8 May 1978, L. Mester et al.: "Heparin Derivatives of high molecular weight", pages 113-120 see the whole document --	1-26	A	US, A, 4239664 (TENG et al.) 16 December 1980 -- ./.	
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>• Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div>																	
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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, " with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	Patent Abstracts of Japan, volume 6, no. 24 (C-091), 12 February 1982 & JP, A, 56147802 (AGENCY OF IND. SCIENCE & TECHNOL), 17 November 1981 -----	

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

CA 9100120
SA 46217

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 08/08/91
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8700060	15-01-87	CH-A- 665954	30-06-88
		EP-A, B 0228387	15-07-87
		JP-T- 63500079	14-01-88
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US-A- 4521564	04-06-85	AU-B- 581831	02-03-89
		AU-A- 3682584	15-08-85
		CA-A- 1221631	12-05-87
		EP-A, B 0152699	28-08-85
		JP-A- 60170617	04-09-85
US-A- 4239664	16-12-80	None	